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A COMPREHENSIVE SURVEY OF BIOLOGICAL MENTHANE PRODUCTION FROM AGRICULTURAL, DOMESTIC AND INDUSTRIAL RESOURCES OF MONTANA

Prepared for

MONTANA DEPAPTMENT of NATURAL RESOURCES and CONSERVATION

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A COMPREHENSIVE SURVEY OF BIOLOGICAL METHANE PRODUCTION FROM AGRICULTURAL, DOMESTIC AND INDUSTRIAL RESOURCES OF MONTANA

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INTRODUCTION

This report concerns studies on anaerobic conversion of various Montana agricultural wastes to methane and on the natural thermophilic microbial processes involved in the anaerobic decomposition of hot spring algal-bacterial mats to methane. Two earlier quarterly reports covered the initial seven months (1/1/78-7/31/78) of the grant period (1/1/78-4/17/79). The last report accompanied a renewal proposal for second year funding which was granted after legislative delay and which initiated on 17 April 1979. Thus, in the period since the last report work has mainly been directed towards the objectives outlined in the renewal grant proposal. In the first grant period we made substantial progress on both topics. The two areas are introduced and discussed separately below.

I. Animal Waste Conversion to Methane

Our initial view of carbon and electron flow in waste conversion to methane is shown in Figure 1. A modified diagram was presented in the last report to indicate the general flow of materials in anaerobic conversion of waste organic matter to methane (Figure 2). Hydrolysis of polymers precedes fermentation reactions leading to the production of methane precursors (H_2 + CO_2 and acetic acid). However, in addition to direct production of methane precursors by fermenting bacteria (Figure 1), these materials are also the products of the oxidation of more reduced volatile fatty acids (propionic, butyric, valeric, isobutyric and isovaleric, but mainly propionic acid) which are produced by fermenting bacteria but which are not directly used by the methane-producing bacteria (Bryant, 1976, 1979; Zehnder, 1978) (Figure 2). It was suggested that these intermediate volatile fatty acids are metabolized by a group termed "obligate H_2-producing acetogenic bacteria" because both H_2 and acetic acid would be products of the proposed catabolic reactions, for example:

$$\text{propionate}^- + 3\ H_2O \longrightarrow HCO_3^- + H^+ + \text{acetate}^- + 3\ H_2 \qquad (1)$$

$$\Delta G^{o\prime} = +18.2 \text{ kcal/reaction}$$

$$\text{butyrate}^- + 2\ H_2O \longrightarrow 2\ \text{acetate}^- + H^+ + 2\ H_2 \qquad (2)$$

$$\Delta G^{o\prime} = +11.5 \text{ kcal/reaction}$$

(equations and free energy changes from Thauer, et al., 1977). Because these reactions are endergonic under standard conditions the existence of such bacteria was postulated to depend on removal of products, as

$$\Delta G' = \Delta G^{o\prime} + RT\ln \frac{\text{products}}{\text{reactants}}$$

(i.e., removal of products lowers the free energy change). Recently, McInerney, et al. (1979) isolated a bacterium capable of performing reaction 2 (as well as anaerobic oxidation of other reduced volatile fatty acids, but not propionate, to H_2 + acetate), but only if a H_2-consuming bacterium such as a methane-producing bacterium, was included as the second member of a coculture. A similar organism capable of performing the thermodynamically more difficult reaction 1 probably also exists based on preliminary evidence (Boone and Smith, 1978; see also Bryant, 1979). These bacteria probably represent the extreme case in which product removal influences carbon and electron flow in natural environments. This has been shown quite clearly in "interspecies H_2 transfer" reactions (Mah, et al., 1977; Wolin, 1974, 1975, 1976).

In natural environments low in sulfate (see below) hydrogen is kept in low concentration by methane-producing bacteria as shown in reaction 3.

$$4\ H_2 + HCO_3^- + H^+ \longrightarrow CH_4 + 3\ H_2O \qquad (3)$$

$$\Delta G^{o\prime} = -32.4 \text{ kcal/reaction}$$

(Thauer, et al., 1977) so that reactions such as 1 and 2 are more likely to proceed. However, in all natural anaerobic environments except the gastrointestinal environment most of the methane produced comes from acetate (reaction 4) and not from $H_2 + CO_2$ (as shown in reaction 3) (Mah, et al., 1977; see also [illegible], et al., 1976 and Winfrey and Zeikus, 1979)

$$\text{acetate}^- + H_2O \longrightarrow HCO_3^- + CH_4 \qquad (4)$$

$$\Delta G^{0\prime} = -7.4 \text{ kcal/reaction}$$

(Thauer, et al., 1977). In anaerobic waste conversion 70-90% of the methane produced comes from acetate (Jeris and McCarty, 1965; Smith and Mah, 1966; Mountfort and Asher, 1978).

It has long been known that failure of anaerobic digesters is associated with imbalance between acid-producing and acid-consuming reactions leading to the accumulation of volatile fatty acids and ultimately to decreased pH (see McCarty, 1964; Zehnder, 1978). Recently, Varel, et al. (1977) showed that with substantial buffering against accumulated volatile fatty acids (e.g., by ammonia produced in large amounts during anaerobic degradation of animal wastes) imbalance between acid-producing and acid-consuming reactions led to very inefficient conversion of waste organic matter to methane. The objectives of our work have revolved around the role of methane-producing bacteria in regulating volatile fatty acid accumulation in the digestion of wastes. In early comparative work we concluded that it was difficult to achieve a balance between acid production and consumption in the fermentation of wheat straw, swine manure and domestic sewage sludge. Thus, our work rapidly focused on anaerobic conversion of dairy cow manure to methane as a model which allowed balanced acid production and consumption to be experimentally unbalanced without pH failure so that the causes for acid accumulation could be investigated. The results described here deal mainly with experiments designed to study the role of H_2- or acetate-consuming methane-producing bacteria in regulating volatile fatty acid removal during the overall conversion of dairy cow manure to methane. A manuscript, recently submitted for publication, is appended. The results presented in Appendix A were also presented by M. Kent Kemmerling at the 1978 Montana Academy of Sciences annual meeting in Dillon.

II. Methanogenesis in Natural Thermophilic Algal-Bacterial Mats

Since 1 January 1979 this aspect of our work has been jointly funded by MDNRC and the National Science Foundation. Studies on thermophilic waste conversion to methane have indicated the biological and economic feasibility of high temperature conversion processes (Varel, et al., 1977; Converse, et al., 1977; Cooney and Wise, 1975; Pfeffer, 1974; Pohland and Bloodgood, 1963), but these studies were on temperature-adapted and not naturally-thermophilic microbial communities. In preliminary work (Ward, 1978) we showed that the algal-bacterial organic matter which forms mats in the effluent channels of hot springs undergoes rapid decomposition to methane. The rationale for our studies on methanogenesis in this environment is to discover how the processes of anaerobic decomposition to methane occur in a natural high temperature environment. Work reported here focused on 1) understanding the earlier observation that optimal methanogenesis occurs at relatively low temperatures in this environment, and on 2) the effect of sulfate on methane production in a high sulfate hot spring. A manuscript, recently submitted for publication, is appended. The results presented as Appendix B were also presented by D. Ward at the 1979 American Society for Microbiology annual meetings in Los Angeles (an abstract is also appended).

METHODS

I. Animal Waste Conversion to Methane

Methods describing the initiation, daily maintenance and monitoring of a dairy cow manure digester are found in Appendix A. Methods for other digesters were similar. The methods employed in subsampling digester contents, performing gas production rate and radiolabelling experiments, and analysing amounts of and/or radioactivity in volatile fatty acids and gases are also given in Appendix A. Enumerations of methanogenic bacteria were done by diluting digester contents ten-fold into preduced sterile medium (after Hungate, 1969) of the following composition: 0.01%(w/v) NH_4Cl, 0.04%(w/v) $K_2HPO_4 \cdot 3H_2O$, 0.01%(w/v) $MgCl_2 \cdot 6H_2O$, 0.0001%(w/v) resazurin, and 0.03%(w/v) $Na_2S \cdot 9H_2O$ (added after autoclaving from a concentrated anoxic stock solution) in distilled water, tubed under helium. The basal medium was supplemented with 1%(w/v) calcium acetate, 1%(v/v) methanol, 0.2%(w/v) trypticase, 0.2%(w/v) yeast extract and/or 10%(v/v) of the liquid obtained after removal of solids by centrifugation of dairy cow manure digester contents, as described below. Hydrogen was provided as 100% H_2 in the gas headspace in place of helium. Final medium pH was 7.4. Incubations were at 36 C. Positive growth of methanogenic bacteria was detected by gas chromatographic analysis of methane in headspace subsamples as described in Appendix A. Direct microscopic observation was made using a Leitz Ortholux II microscope equipped for vertical illumination fluorescence microscopy using an HBO-200 watt mercury ultraviolet light source and a B-cube combination of excitation and emission filters (Leitz).

II. Methanogenesis in Natural Thermophilic Algal-Bacterial Mats

Methods for work on natural high temperature algal-bacterial mats were described by Ward (1978) or are described in Appendix B.

RESULTS AND DISCUSSION

Results presented in this section are intended to reflect the natural progression of experimental approaches all directed at examination of the role of methane production reactions in regulation of carbon and electron flow. Artificial increases in carbon and electron flow indicated that methane production reactions may become saturated leading to acetate increase and also to accumulation of propionate and other reduced volatile fatty acids. The kinetics of acetate conversion to methane were examined to indicate the potential for saturation of that methane production reaction. Also, the effects of the substrates of methane production reactions which would accumulate on saturation (i.e., H_2 and acetate) on propionate degradation were studied to observe the role of methane production reactions in regulating turnover of reduced volatile fatty acids. Many experiments are described in greater detail in an appended manuscript (Appendix A).

I. Animal Waste Conversion to Methane

A. Comparison of methane fermentation in Montana wastes.

We attempted to achieve a stable fermentations of dairy cow manure, swine manure, domestic sewage sludge and wheat straw to methane. Dairy cow manure conversion to methane was easily established at a 15 day turnover time after an initial enrichment of methanogenesis (about two weeks) followed by daily addition (fresh manure) and removal (digester contents) of 1/15th of the total volume. Relatively stable pH, gas production rate, and gas composition (see Table 1) and low volatile fatty acid concentrations (see Table 2) were readily established. Swine waste and domestic sewage sludge methane fermentations were more difficult to establish and required longer initial turnover times to accomplish stable fermentation conditions (see Table 1). Higher volatile fatty acid levels and a higher propionate/acetate ratio indicated that the balance between acid consumption and production was not as good in these digesters (see Table 2). Similar difficulties in the initiation of swine waste conversion digesters were reported previously (Schmid and Lipper, 1969; Hobson and Shaw, 1973; Lapp, et al., 1975). The association of volatile fatty acid accumulation and domestic waste digester failure has long been noted (McCarty, 1964; Zehnder, 1978). We were unable to obtain a stable methane fermemtation of wheat straw. Despite efforts to seed the wheat straw digester with contents of the dairy cow manure digester, stabilization of pH required constant addition of alkali (KOH) and gas production was always very low (see Table 1). Because of the greater stability of dairy cow manure conversion to methane, further experimentation was done using this waste as a model in which to study the influence of methanogenic bacteria on carbon and electron flow.

B. Experiments on dairy cow manure conversion to methane.

1. Relative importance of methane production reactions.

The importance of reaction 3 in methane production can be determined by comparing specific activities (SA) of carbon dioxide and methane after addition of $NaH^{14}CO_3$. Because of the difference in pathways of methane produced by reactions 3 and 4 (i.e., methane is produced only by direct reduction of the methyl carbon of acetate and not via carbon dioxide as an intermediate (see Mah, et al., 1977)) methane produced from acetate in the same time interval would not be radioactive and would thus dilute the methane specific activity compared to the specific activity of carbon dioxide. The ratio of SA_{CH_4}/SA_{CO_2} represents the fraction of methane produced from reaction 3. Although other methane precursors could contribute to methane production via reactions other than 3 and 4 (for example, methanol (see Mah, et al., 1977, methyl amine (see Weimer and Zeikus, 1978a), acetate is probably the only other significant methane precursor in waste conversion systems (as measured by comparison of the rates of acetate turnover and methane production, see Smith and Mah, 1966; Mountfort and Asher, 1978). In our digester the relative importance of reaction 3 in methane production was 28.8% ± 8.4% standard deviation (mean of seven determinations) when measured 2.5-9.5 hours after daily feeding. In two experiments we observed that the relative importance of reaction 3 increased to 39.4% ± 4.5% standard deviation (mean of eight determinations) when measured 27-56.5 hours following daily feeding of fresh manure. The acetate concentration was observed to increase after feeding (in comparison to 12 hours after feeding, see Table 2). The immediate increase was probably due to acetate in the added feed (see Table 3) which should have increased the acetate concentration by 2.9 mM considering a 1/15 dilution of the acetate concentration in fresh manure (observed difference between 0 and 12 hours after feeding was 3.2 mM). If reaction 4 is undersaturated (see below), addition of fresh manure and the coincident increase in acetate should increase the rate of reaction 4 relative to reaction 3 so that the relative importance of reaction 3 would be lower until the acetate concentration was lowered by metabolism. This was, in fact, what we observed. Mountfort and Asher (1978) reported similar findings in a bovine waste digester. As in other waste conversion systems acetate was found to be the main methane precursor (Jeris and McCarty, 1965; Smith and Mah, 1966; Mountfort and Asher, 1978). The shifting relative importance of methane production reactions, as well as other parameters (such as changes in gas production rate (see Figure 3) and volatile fatty acid levels (see Table 2) following daily feeding), indicated the semi-continuous nature of the digester which was fed once daily.

2. Increased carbon and electron flow to methane production reactions.

The flow of carbon and electrons to acid-consuming reactions was artificially increased by addition of glucose to mimic accelerated hydrolysis of polymers (see Figure 2). As metabolism of added glucose led to the production of volatile fatty acids but not to increased production of the natural buffer ammonia, excessive glucose loading resulted in pH decrease and digester failure. The accumulation of acetate and propionate and drop in pH in response to addition of 5% glucose is shown in Figure 4. Some time is required for metabolism of added glucose to result in an increase in volatile fatty acids, and for the increase in volatile fatty acids to result in lowered pH as acidity exceeds the buffering capacity. Tolerance to various levels of added glucose is shown in Table 4. Addition of 1% or 2.9% glucose did not result in pH lowering even after 33 hours (Table 4). However, addition of 4.7% or higher glucose amounts eventually caused digester failure. Additions of 1% or 2.9% glucose led to very high acetate (27.4, 32.1 mM, respectively), propionate (20.8, 14.6 mM) and butyrate (1.6, 7.3 mM) levels relative to unsupplemented control levels (1.6 mM acetate, 1.3 mM propionate, undetectable butyrate). Lower levels of these volatile fatty acids were noted 33 hours after addition of higher amounts of glucose, but this was presumably due to the rapid decrease in pH and volatile losses of the fatty acids during the rapid out-gassing which followed glucose addition (see Figures 3 and 4). The accumulation of acetate suggested that increased carbon and electron flow associated with increased hydrolysis of polymeric material fed (mimicked by glucose addition) may lead to saturation of acetate consumption reactions such as reaction 4. The increase in propionate relative to acetate was shown in a separate experiment in which 5% glucose was added (Figure 5). The increasing propionate to acetate ratio indicated the possible inhibition of reaction 1 because of increased levels of products (i.e., H_2 and/or acetate). Acetate and propionate accumulations were observed in other waste conversion systems when carbon and electron flow was increased by addition of monomeric or polymeric organic carbon (Sykes, 1970) or by increases in feed concentration or decreases in turnover time (Varel, et al., 1977).

Several experiments were attempted to test the hypothesis that volatile fatty acid accumulation was related to saturation of reaction 4 (i.e., acetate conversion to methane). As methane is produced by both reactions 3 and 4, the saturation of one reaction before the other should lead to an increasing importance of the other reaction in methanogenesis (as reported above for changes following addition of acetate in the feed). If, as postulated, glucose addition led to saturation of reaction 4 (but not reaction 3), as suggested by increases in acetate, the relative importance of reaction 3 should be increased. Glucose addition, however, was found to have no repeatable effect on the relative importance of reaction 3, indicating that both reactions 3 and 4 may be similarly affected by increased carbon and electron flow.

3. Kinetics of acetate conversion to methane under stable operating conditions.

Stimulation of gas production rate by added sodium acetate (see Figure 3) indicated that reaction 4 was undersaturated during normal digester operation. It was of interest to examine the dependence of reaction 4 on acetate concentration to establish the acetate level required for saturation. A preliminary indication of the dependence of the reaction on acetate concentration was given when the increase in gas production rate (presumably due to reaction 4) was related to added sodium acetate (Figure 1B or Appendix A). Gas production rate was increased by acetate to about 20 mM levels, but further acetate addition did not increase gas production rate. Addition of sodium acetate in amounts sufficient to saturate gas production (i.e., 20 mM)(at varying times after feeding of fresh manure) was less stimulatory soon after feeding than at substantial times after feeding (Figure 6). This observation may be related to the increase in acetate (Table 2) and corresponding increase in gas production rate (Figure 3) following feeding. Further additions of acetate to a larger acetate pool would cause rapid saturation of reaction 4 and would stimulate gas production to a lesser degree. When reaction 4 was studied directly by addition of 2-^{14}C-acetate and measurement of the rate of $^{14}CH_4$ production saturation was observed at about 20 mM acetate (Figure 1A of Appendix A; see Appendix A for details of this experiment). As the indigenous acetate level was in the 4-8 mM range (see Table 2) following normal daily feeding, reaction 4 was about half saturated under typical operating conditions. Increased carbon and electron flow on glucose addition led to acetate concentrations sufficient to saturate the reaction (see above).

It was not possible to examine the kinetics of reaction 3, the other important methane production reaction, as it was impossible to quantitate the H_2 concentration. However, increased carbon and electron flow on glucose addition did not alter the relative importance of reaction 3 (see above), suggesting that both reactions 3 and 4 may be proportionately increased as pool levels of acetate and H_2 are elevated, leading to increases in methane production by both reactions to the same extent.

4. Propionate degradation.

Coincident propionate and acetate accumulation on glucose addition suggested that acetate (and H_2) may accumulate to make propionate degradation thermodynamically difficult. In order to assess the effect of H_2 and acetate on propionate degradation, it was necessary to investigate the mechanism of propionate oxidation in our system. The rapid production of $^{14}CO_2$ from 1-^{14}C-propionate but not from 3-^{14}C-propionate was consistent with reaction 1 as the mechanism of propionate degradation (Figure 2 of Appendix A). Further evidence came from the recovery of 1-^{14}C-propionate as unmetabolized propionate and not as ^{14}C-acetate (by combined gas chromatography--gas proportion analysis). Details of these methods and experiments are provided in Appendix A.

5. Effect of acetate and H_2 on propionate decarboxylation.

Reaction 1 was studied by following the rate of 1-^{14}C-propionate conversion to $^{14}CO_2$. Acetate increasingly inhibited the rate of reaction 1 as various salts of acetate decreased the reaction rate, whereas chloride slats had no inhibitory effect (see Figures 3-5 of Appendix A). Hydrogen also inhibited reaction 1, although the extent of inhibition could not be quantified because the effect of addition of H_2 to the gas headspace on the H_2 pool level was unknown (see Figure 6 of Appendix A). Neither 500 mM acetate nor 70% H_2 in the gas headspace completely inhibited reaction 1. Thus, significant inhibition of propionate degradation was observed by artificial increases in both acetate and H_2, suggesting that accumulation of these methane precursors upon saturation of reaction 3 and 4 could have a negative influence on propionate turnover, leading to its increase. Acetate addition to 50 mM also resulted in a threefold increase in propionate concentration measured 12 hours after acetate addition. Similar results indicating the inhibition of propionate turnover and the accumulation of propionate by acetate or hydrogen additions to digesting sewage sludge were reported during the grant period (Kaspar and Wuhrmann, 1978a,b).

6. Methanogenic bacteria.

Enumeration of the methanogenic bacterial population of digesting dairy cow manure was made using hydrogen or acetate as energy sources for methane-producing bacteria. Methanol was also provided as an energy source in some cases because of anticipated problems with enumeration using acetate and because both methanol and acetate are used by the known acetate-utilizing methanogenic bacterium. The basic medium was supplemented in some cases with cow manure digester extract or trypticase plus yeast extract to provide growth factors, as the only known acetate-using methanogenic bacterium, Methanosarcina barkeri, has undefined growth factor requirements (Smith and Mah, 1978; Mah, et al., 1978; Weimer and Zeikus, 1978b). As noted in Table 5, 10^9 methanogenic bacteria per milliliter were detected on the most complex medium using H_2 as the energy source. As all known methanogenic bacteria use H_2 (Bryant, 1974), this number is probably representative of the total methanogenic population density. The recovery of methanogenic bacteria using methanol or acetate as energy sources was not as good (10^4-10^6 cells/ml). Because of the relative importance of reaction 4 in methanogenesis in our digester this result seems inconsistent. However, our findings are similar to those of others (Hobson, et al., 1974) and presumably reflect the difficulty in enumerating acetate-consuming methanogenic bacteria. Direct observations by fluorescence microscopy indicated a predominance of large cocci and occasional clumps of sarcinae, as well as rod-shaped bacteria which exhibited blue-green autofluorescence. This was presumptive evidence of a greater abundance of methanosarcinae (than indicated in Table 5), detected because of the common presence of the autofluorescing coenzyme F_{420} in all known methanogenic bacteria (Mink and Dugan, 1977). Problems in enumeration of methanosarcinae by dilution techniques may relate to the physical problem of disrupting a clumping organism. *Methanosarcina barkeri* is the only methanogenic bacterium described which is capable of acetate conversion to methane (Balch, et al., 1979; Mah, et al., 1977; Bryant, 1974).

II. Methanogenesis in Natural Thermophilic Algal-Bacterial Mats

A. Methane Production in Alkaline Silicious Hot Springs

1. Temperature optimum for methanogenesis.

Our major effort in the 1978 field season was to confirm the temperature distribution of methanogenesis found previously in Octopus Spring (Ward, 1978) by studying methanogenesis in several Yellowstone hot spring algal mats (Figure 7). Springs studied in the Great Fountain Geyser area were Octopus, Twin Butte Vista and Mushroom springs and an artificial channel constructed from a spring in the Serendipity springs group by Dr. Dick Weigert of the University of Georgia. Methanogenesis in Twin Butte Vista and Mushroom spring thermal gradients showed optima at 55 and 60 C, respectively. These optima were higher than the optimum at 45-50 C at Octopus spring (data of Ward, 1978 and Figure 7). Of extreme interest was the finding that methanogenesis in the Weigert channel thermal gradient, although optimal at 55 C, was very rapid at 60 and 65 C. This was in marked contrast to the other springs where no methanogenesis occurred at 65 C and where methanogenesis at 60 C occurred at only very slow rates. Microbiologically, there is a striking difference between the Weigert channel and the algal-bacterial mats of other spring effluents, as it is comprised in part of the blue green alga Mastigocladus laminosum in addition to the blue green alga Synechococcus lividus and the photosynthetic bacterium Chloroflexus aurantiacus. The basis for this difference is not known though the level of combined nitrogen in the spring waters may be very influential as Mastogocladus is capable of N_2 fixation (the enzymes for this process are repressed by fixed nitrogen and when repressed might alter the competitiveness of the alga). It is interesting to speculate that N_2 fixation may be related to increased methanogenesis at 60 and 65 C in the Weigert channel. However, the upper temperature limit of 58-60 C for Mastogocladus laminosum raises doubt about this hypothesis. We made an effort to find a spring in the West Thumb group, which Weigert (personal communication) described as containing Mastigocladus laminosum, in order to obtain correlative evidence that high rates of methanogenesis at elevated temperatures are found in algal-bacterial mats containing this organism. The spring we studied did not contain Mastigocladus and had low or no methanogenesis at 60 and 65 C (see Figure 7). In all five springs studied the temperature optimum for methane production was lower than the upper temperature limit of algal-bacterial mat growth (i.e., about 70 C) by at least 10-15 C.

2. Adaptation to temperature by bacteria involved in anaerobic decomposition.

The adaptation of some of the organisms involved in mat growth and degradation to temperature was shown previously (see Brock, 1978). We examined the possibility that methanogenic bacteria (or other bacteria involved in anaerobic decomposition to methane, as measurements of methane production depend on the cooperative functioning of bacteria involved in prior steps of decomposition of organic matter to produce the substrates of methanogenic bacteria,(see Figure 2) were unable to function at temperatures higher than the temperature of optimal methanogenesis observed in the natural environment. Experiments were performed in Octopus Spring where the temperature optimum for methanogenesis was 45-50 C. Samples from various temperatures along the thermal gradient were incubated at temperatures higher and lower than the indigenous temperature of the sample. As shown in Figure 8 the temperature relations of bacteria involved in conversion of the algal-bacterial organic matter to methane were similar for samples collected at all temperatures along the thermal gradient. Methane production was increased by increasing temperature and was maximal at the highest incubation temperature tested (65 C). Similar results were observed for the algal-bacterial mat at Weigert's channel where the temperature optimum for methanogenesis was 55 C (see Figure 9). Since the anaerobic degradation to methane was higher at higher temperatures (i.e., higher temperatures did not inhibit methanogenesis) the restriction of maximal methanogenesis to suboptimal temperature must be based on other factors than temperature. As the anaerobic decomposition processes along the thermal gradient depend on the rate of production of algal-bacterial biomass, it is likely that restriction of primary production to lower temperatures may restrict anaerobic degradation and thus methanogenesis to suboptimal temperatures (Ward, 1978; Doemel and Brock, 1977).

3. Metabolism of methane precursors.

Figure 2 serves as a useful model for anaerobic decomposition of organic matter in low sulfate environments other than animal digestive tracts where acetate is consumed in the energy metabolism of the host animal (Bryant, 1979). It was initially assumed that carbon and electron flow was similar in the decomposition of low sulfate hot spring algal-bacterial mats. Preliminary experiments were performed on mat samples collected from a 50 C region of Twin Butte Vista spring. Rapid reduction of $NaH^{14}CO_3$ to $^{14}CH_4$ indicated the presence of organisms capable of catalyzing reaction 3. However, conversion of $2\text{-}^{14}C$-acetate to $^{14}CO_2$ and not to $^{14}CH_4$ raised doubt about the participation of reaction 4 in methane production in this environment. Although we previously reported (last quarterly report) that dilution of specific activity in $NaH^{14}CO_3$ labelling experiments suggested that other methane production reaction than reaction 3 must be operative, our confidence in specific activity calculations in these experiments has decreased as a result of subsequent work. The lack of $^{14}CH_4$ production from $2\text{-}^{14}C$-acetate is presumptive evidence that acetate may not be an important precursor to methane in this environment. Of immediate interest is how acetate production in anaerobic fermentation of algal-bacterial organic matter is balanced, in the absence of reaction 4, to prevent acidification and failure of the overall anaerobic conversion process.

B. Anaerobic Decomposition Processes in a High Sulfate Hot Spring

Studies at Bath Lake in the Mammoth Terrace group at Yellowstone National Park were performed on methane production and sulfate reduction in the anaerobic decomposition of an algal-bacterial mat overlying the sediment. The mat at 40 C is primarily comprised of the blue green algae *Synechococcus lividus*, *Synechococcus minervae*, and *Spirulina labyrinthiformis*, and the photosynthetic bacterium *Chloroflexus aurantiacus* (Castenholz, 1977). The high sulfate level of this spring (700-800 mg $SO_4^{=}$/liter) compared with low sulfate alkaline silicious springs (see Table 6) provided a natural high sulfate environment in which to study the roles of methane-producing and sulfate-reducing bacteria in the anaerobic decomposition of algal-bacterial organic matter.

Sulfate reduction, and not methane production, was the predominant terminal process in the degradation of algal-bacterial organic matter in this environment. This was suggested by the rapid depletion of sulfate and generation of sulfide coincident with production of carbon dioxide on anaerobic incubation of cores of the Bath Lake mat (Figure 1 of Appendix B). Although it appeared that methane production did not occur until after sulfate depletion (over 200 hours after sampling), more sensitive measurements indicated slow, but detectable increases in methane during the period of sulfate reduction (Figure 2 of Appendix B). Further evidence for the dominance of sulfate reduction came from the lack of conversion of ^{14}C-labelled methane precursors (i.e., $NaH^{14}CO_3$ and 2-^{14}C-acetate) to $^{14}CH_4$ and the rapid oxidation of 2-^{14}C-acetate to $^{14}CO_2$ (consistent with the activity of acetate-oxidizing sulfate-reducing bacteria (Widdel and Pfennig, 1977)) (see Figure 3 of Appendix B). Also, radiolabelled sulfate (i.e., $Na^{35}SO_4$) was rapidly reduced to $H_2{}^{35}S$ (Figure 4 of Appendix B) in the upper layers of the algal-bacterial mat. When the initial rates of sulfate reduction and methane production were compared, sulfate reduction was found to be at least 1000 times more significant (see Appendix B). It is also important to note that methane production is probably never significant in this system because depth profiles showed no sulfate depletion in the sediment (Figure 6 of Appendix B). Radiolabelling experiments conducted on different depth intervals showed the dominance of sulfate reduction at all depth intervals (Table 1 of Appendix B). The severe limitation of methane production by sulfate has been observed in other environments (see Mah, et al., 1977). Recently Winfrey and Zeikus (1977) showed that competition for the common energy sources acetate and H_2 is probably the basis for this inhibition. Further details and discussion of these experiments is given in the appended manuscript.

CONCLUSIONS

I. Animal Waste Conversion to Methane

In the first grant period we have focused on the conversion of dairy cow manure to methane as a model for stable efficient waste conversion. Stable, but less efficient, methane fermentations of swine manure and domestic sewage sludge were achieved. Wheat straw was not a suitable material for anaerobic conversion to methane. Under normal operating conditions (15 day turnover of 5.1% dry weight solids) dairy cow manure was efficiently converted to methane (Figure 10A). Methane production appeared to occur via carbon dioxide reduction but predominantly by conversion of acetate to methane. Under normal operating conditions acetate concentration was in a range sufficient to only half saturate acetate conversion to methane. Thus, under the operating conditions selected methanogenic bacteria catalyzing the predominant methane production reaction were not saturated for their energy source and did not limit the overall rate of waste conversion to methane (i.e., acetate consumption was more rapid than its formation). Addition of glucose mimicked acceleration of polymer hydrolysis (the presumed rate-limiting step under the chosen operating conditions) and thus, the flow of carbon and electrons to methane production reactions (Figure 10B). The resultant increase in acetate was sufficient to saturate the conversion of acetate to methane and, thus, to make this (and probably other) methane production reactions rate-limiting for the overall conversion of organic matter to methane. Coincident accumulation of propionate (and other reduced volatile fatty acids) suggested the possibility that the accumulated methanogenic substrate acetate (and possibly H_2)--the products of the thermodynamically difficult oxidations of the more reduced fatty acids (e.g., propionate, butyrate)--could inhibit the turnover of volatile fatty acids. The potential for H_2 and acetate inhibition of propionate decarboxylation--the predominant mechanism of propionate degradation in our digester--was demonstrated. Under loading conditions which did not exceed buffering capacity the accumulation of volatile fatty acids decreased the overall efficiency of organic matter conversion to methane. Under loading conditions which exceeded the buffering capacity, digester failure resulted from a dramatic decrease in pH following accumulation of volatile fatty acids. Although stable efficient conversion of dairy cow manure to methane is possible, increases in conversion rates and efficiency to maximum are desirable. Our observations suggest that improvements in release of polymeric organic matter (such as delignification or increased manure loading) or attempts to maximize conversion rates (such as decreased turnover time) will lead to rate limitation of waste conversion to methane by saturation of methanogenic reactions. Accumulation of methanogenic substrates may inhibit degradation of other volatile fatty acids. The result will be decreased conversion efficiency or failure of the fermentation as carbon and electrons are diverted from methane production to acid accumulation. The methane-producing bacteria thus play a critical role in maintaining stability and efficiency of digester operation. Further research is needed 1) to verify the role of methane-producing bacteria in regulating carbon and electron flow under more natural conditions of stress (such as increased loading rates and decreased turnover time) rather than under the artificial conditions imposed by glucose addition, and 2) to explore the population responses of methanogenic bacteria to increased production of methane precursors which may lead to long term adjustment to more stressed operating conditions.

II. Methanogenesis in Natural Thermophilic Algal-Bacterial Mats.

In the first study period substantial progress was made toward understanding the ecology of methane-producing bacteria in natural thermal environments. The observation that maximal rates of methane production occur at temperatures well below the upper temperature of the algal-bacterial mats was made on five different hot springs. Higher rates of methane production in one spring raised the possibility that nutrient limitation might be the basis for this observation. Inhibition of anaerobic conversion processes by high temperature was not the basis for restriction of maximal methanogenesis to lower temperature as increases in temperature stimulated methanogenic rates in samples collected from all temperature regions of the mat. As anaerobic degradation in this environment is limited by the rate of production of algal-bacterial organic matter (i.e., photosynthesis) we hypothesize that limitations in primary productivity may restrict mat formation and anaerobic degradation to methane to lower temperatures along the thermal gradient. Methane production appears to occur mainly via carbon dioxide reduction. The mechanism for acetate removal to prevent acid accumulation is apparantly different from other natural environments where acetate consumption by methanogenic bacteria predominates. Methane production was insignificant compared to sulfate reduction as a terminal process involved in anaerobic degradation of algal-bacterial organic matter in a high sulfate hot spring. The inhibition of methane production appears, as in other high sulfate environments, to be based on competition for the common energy sources H_2 and acetate. Further work is needed 1) to confirm the hypothesis that the temperature distribution of methane production in low sulfate mats is caused by restriction of photosynthetic activity to this thermal regime, and

2) to examine the role of the various methane production reactions involved in anaerobic conversion of organic matter to methane in low sulfate hot springs.

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FIGURE 1. INITIAL VIEW OF REACTIONS INVOLVED IN CONVERSION OF WASTE ORGANIC MATTER TO METHANE

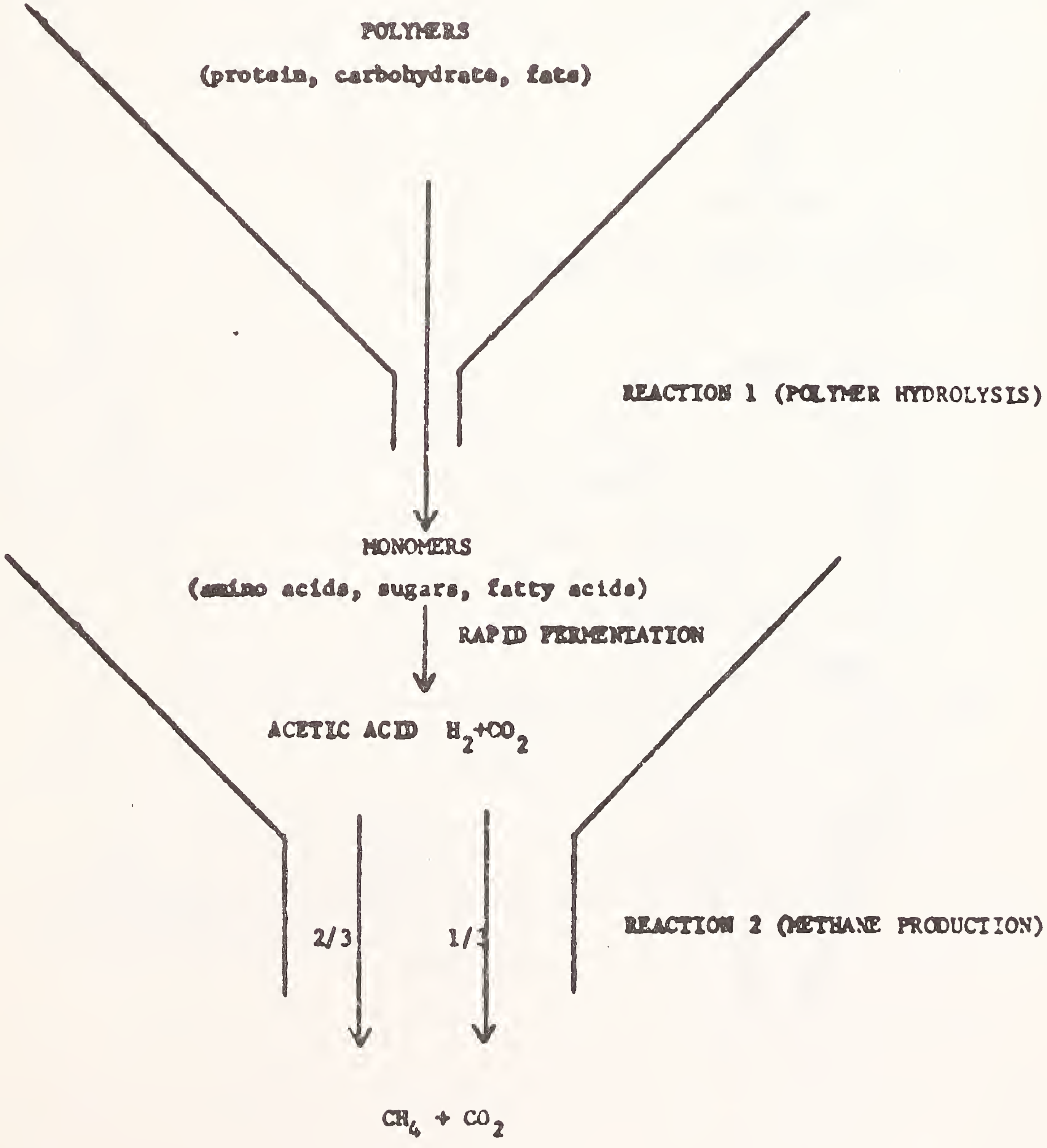
POLYMERS
(protein, carbohydrate, fats)
REACTION 1 (POLYMER HYDROLYSIS)
MONOMERS
(amino acids, sugars, fatty acids)
RAPID FERMENTATION
ACETIC ACID H_2+CO_2
2/3
1/3
REACTION 2 (METHANE PRODUCTION)
CH_4 + CO_2

FIGURE 2. DETAILED VIEW OF REACTIONS INVOLVED IN ANIMAL WASTE CONVERSION TO METHANE

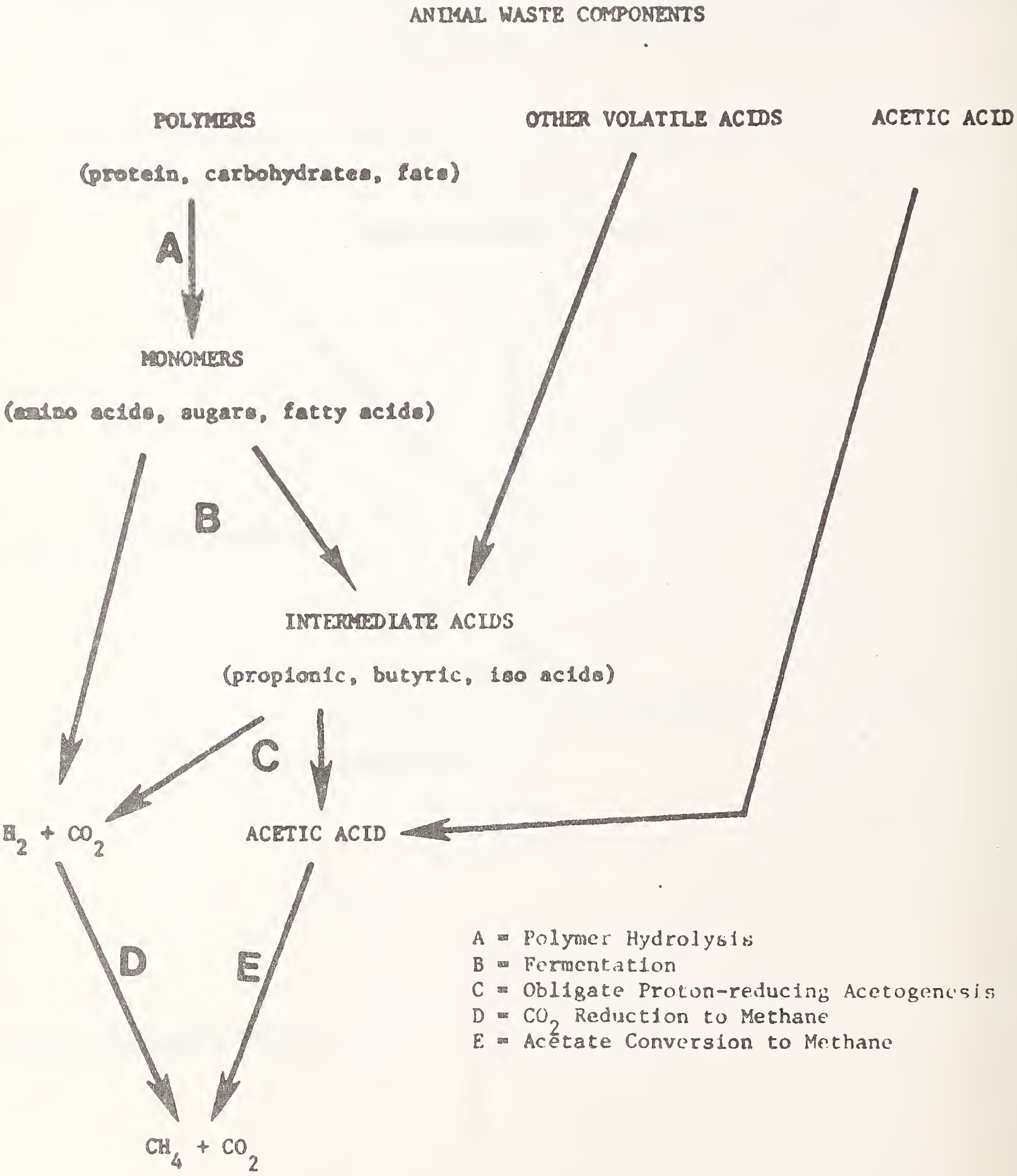
ANIMAL WASTE COMPONENTS
POLYMERS
(protein, carbohydrates, fats)
OTHER VOLATILE ACIDS
ACETIC ACID
A
MONOMERS
(amino acids, sugars, fatty acids)
B
INTERMEDIATE ACIDS
(propionic, butyric, iso acids)
C
$H_2 + CO_2$
ACETIC ACID
D
E
$CH_4 + CO_2$
A = Polymer Hydrolysis
B = Fermentation
C = Obligate Proton-reducing Acetogenesis
D = CO_2 Reduction to Methane
E = Acetate Conversion to Methane

FIGURE 3. EFFECT OF VARIOUS ADDITIONS ON GAS PRODUCTION RATE AND COMPOSITION IN A DAIRY COW MANURE DIGESTER

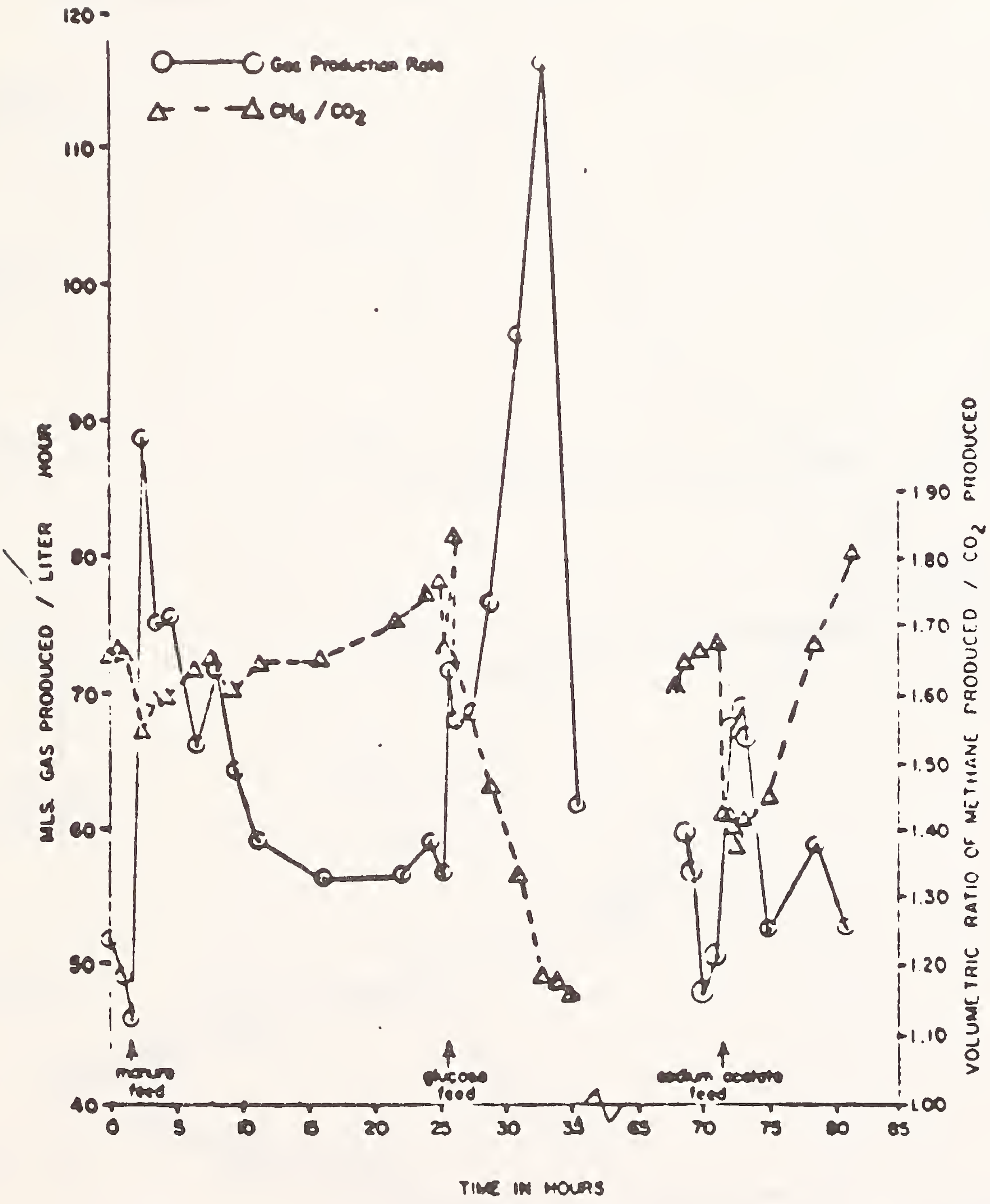

FIGURE 4. RESPONSES TO THE ADDITION OF 5% GLUCOSE TO A DAIRY COW MANURE DIGESTER

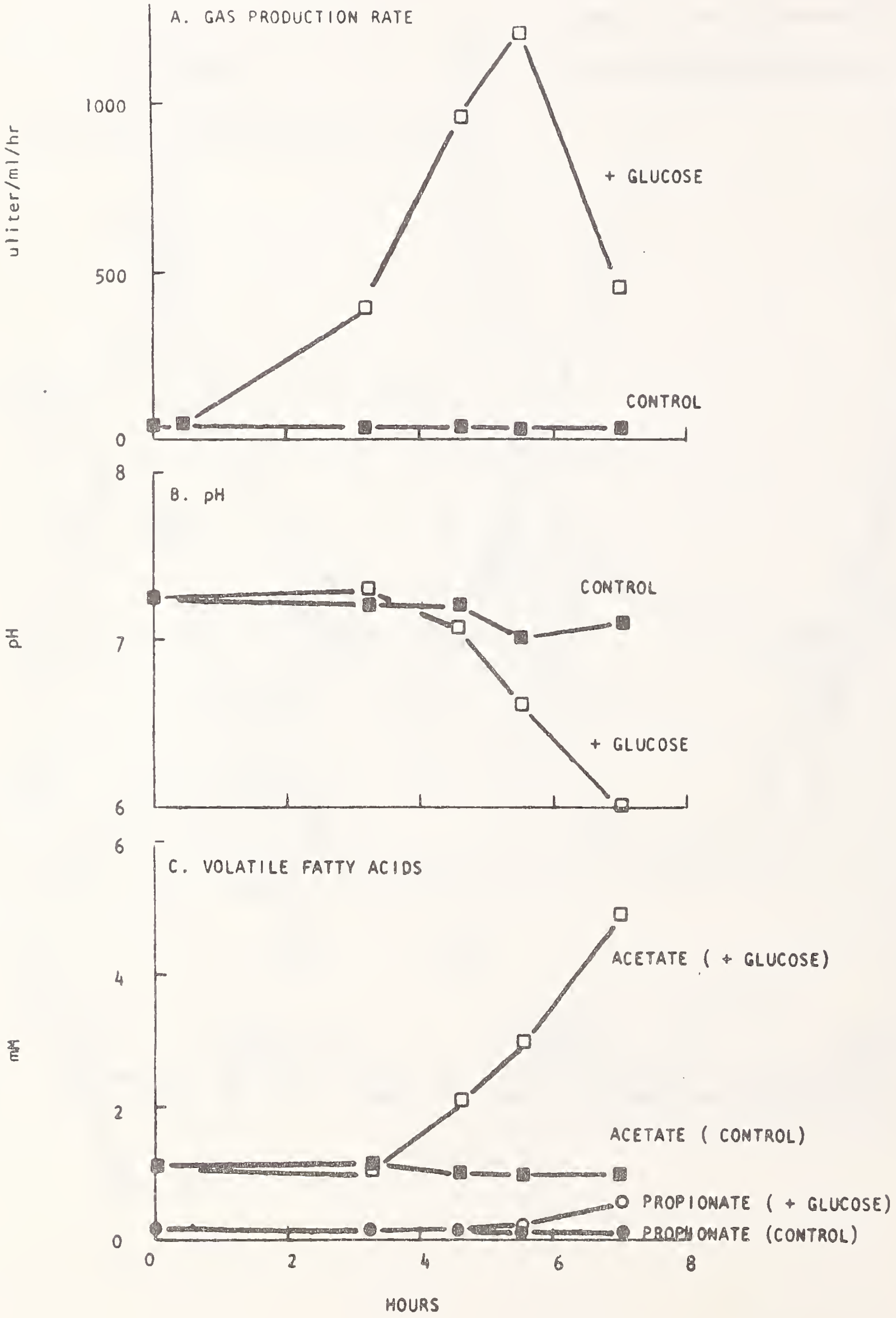

FIGURE 5. EFFECT OF GLUCOSE ON PROPIONATE/ACETATE RATIO IN A DAIRY COW MANURE DIGESTER

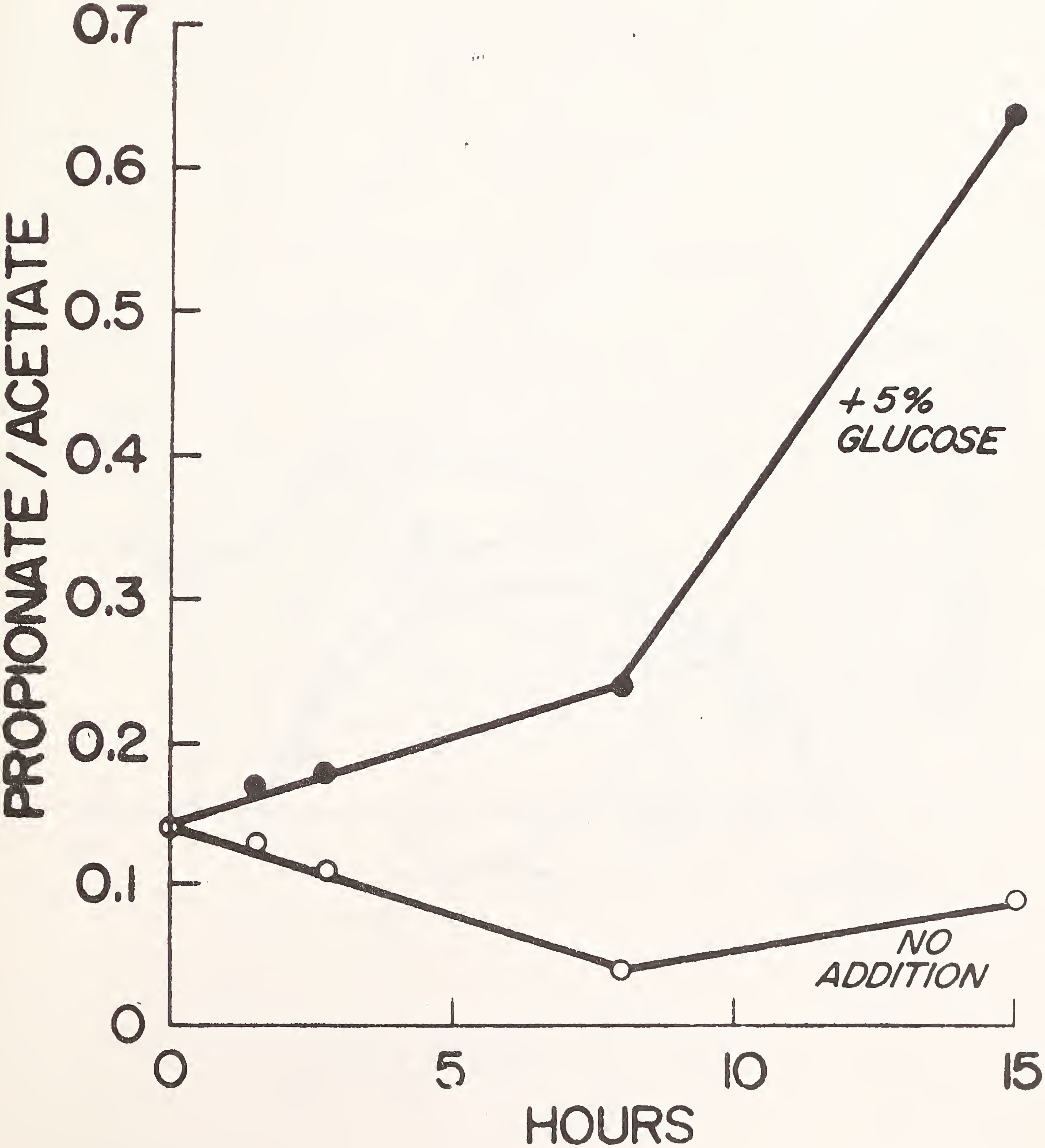

FIGURE 6. EFFECT OF ADDITION OF SODIUM ACETATE(TO 20 mM) ON GAS PRODUCTION RATE AT VARYING TIMES AFTER DAILY FEEDING OF A DAIRY COW MANURE DIGESTER

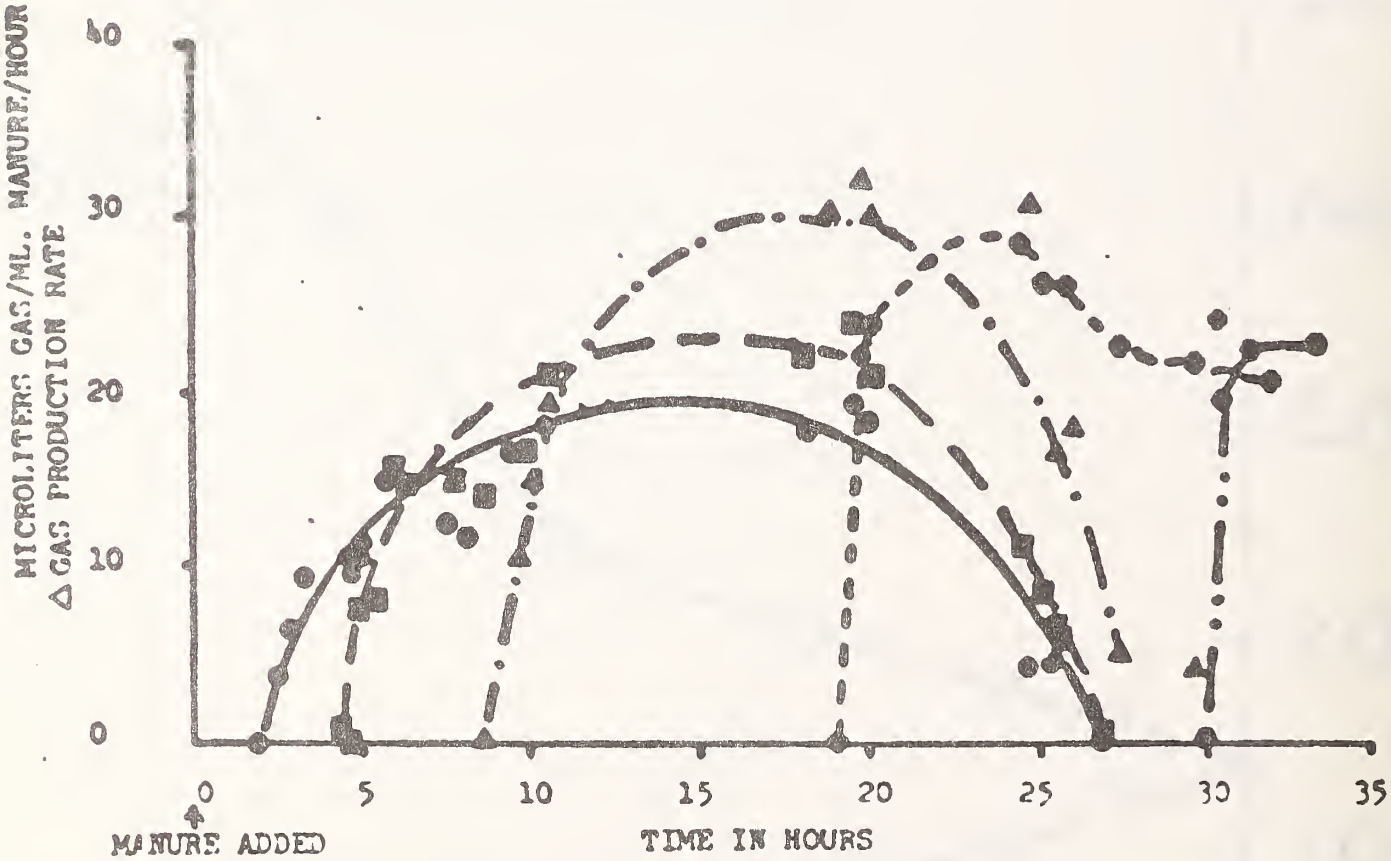

FIGURE 7. TEMPERATURE DISTRIBUTION OF METHANE PRODUCTION (17-20 hours) IN ALGAL-BACTERIAL MATS OF SEVERAL YELLOWSTONE HOT SPRING THERMAL EFFLUENTS

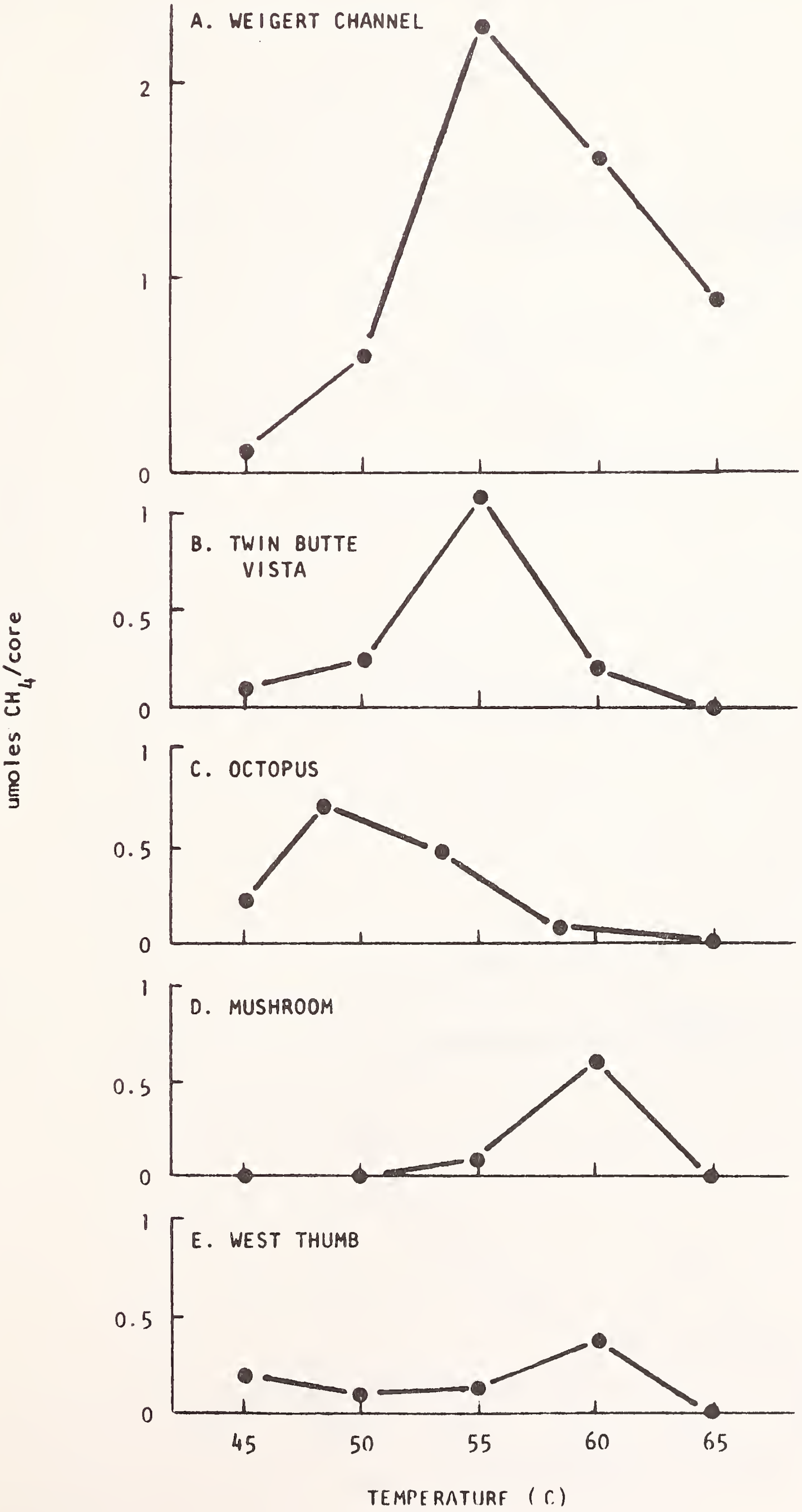

FIGURE 8. EFFECT OF TEMPERATURE ON METHANOGENESIS IN OCTOPUS SPRING ALGAL-BACTERIAL MAT MATERIAL COLLECTED AT VARIOUS TEMPERATURES

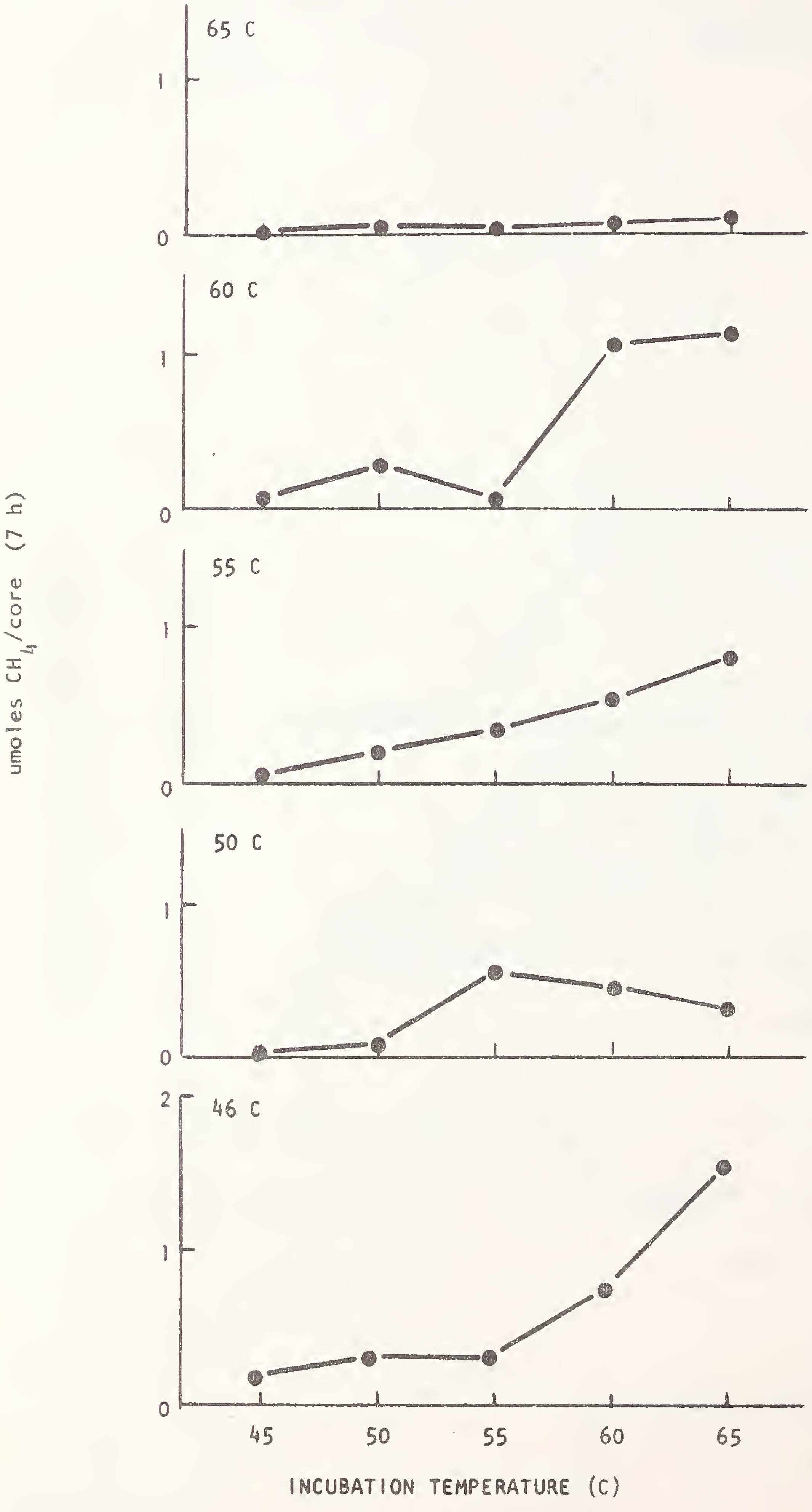

FIGURE 9. EFFECT OF TEMPERATURE ON METHANOGENESIS IN WEIGERT CHANNEL ALGAL-BACTERIAL MAT MATERIAL COLLECTED AT VARIOUS TEMPERATURES

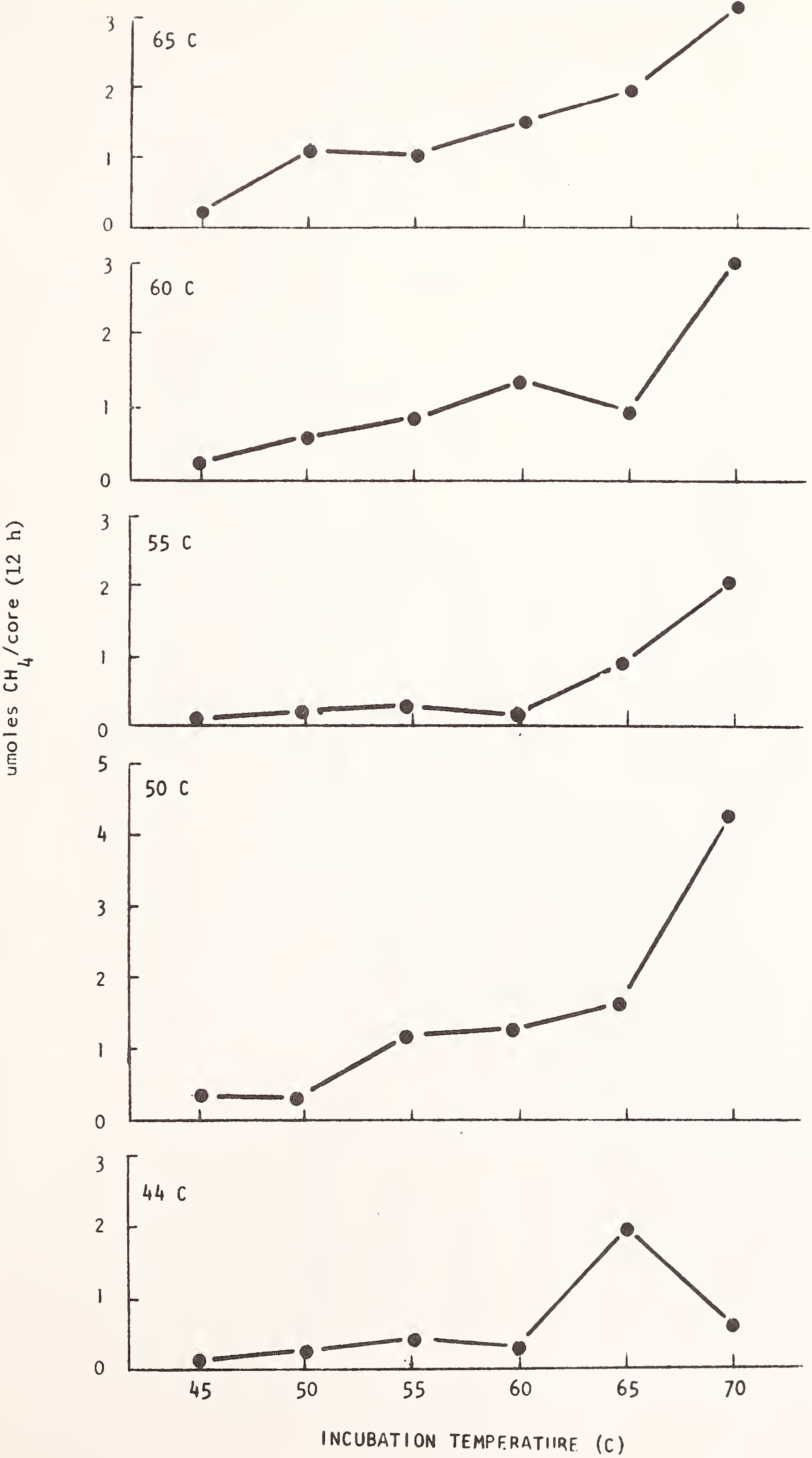

Figure 10 Models for carbon and electron flow in dairy cow manure conversion to methane under A) balanced and B) unbalanced conditions. A slash through an arrow indicates saturation. Dashed lines indicate potential inhibition or saturation of reactions.

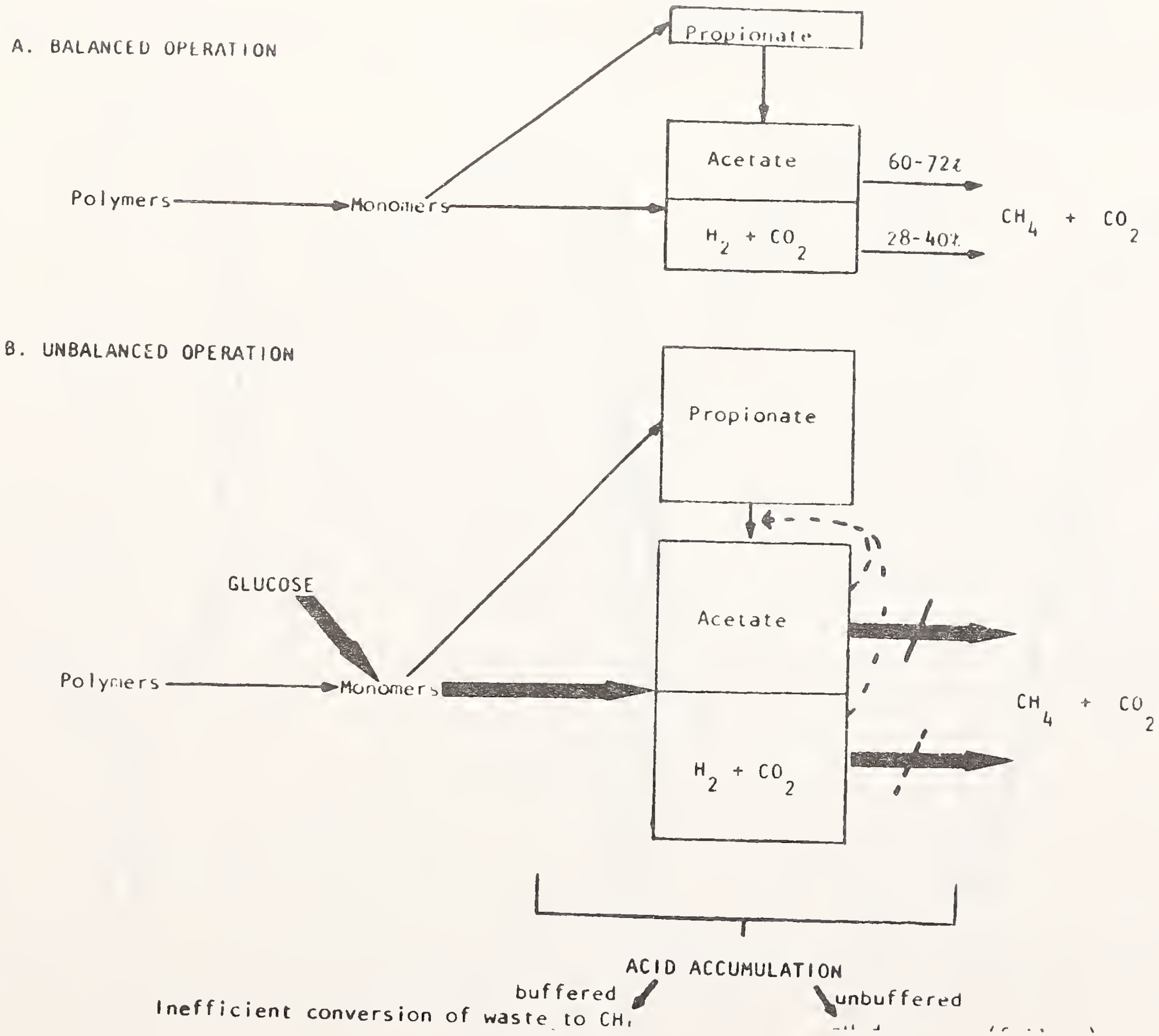

TABLE 1. OPERATING CONDITIONS IN WASTE DIGESTERS

Waste Material	Feed Concentration gm dry wght/100 ml	Turnover time	pH	$\frac{\text{ml gas produced}}{\frac{\text{liter waste}}{\text{hour}}}$	Average %CH_4
Dairy Cow Manure	5.1	15 days	7.6	60	53
Swine Manure	9.3	45 days	8.2	40	63
Domestic	8.0	15 days[a]	6.9	29.4	62
Wheat Straw	4.0	b	7.3[c]	9.3	49

a, recently shifted from 45 day turnover time

b, no regular addition of feed was attempted as the digester never initially acclimated

c, pH was constantly buffered by the addition of KOH and was highly unstable otherwise

TABLE 2. VOLATILE FATTY ACID LEVELS IN WASTE DIGESTERS

Waste Material	Interval after feeding	pH	mmoles/liter						total
			acetic	propionic	isobutyric	butyric	isovaleric	valeric	meqacid/liter
Dairy Cow Manure	immediate	7.6	7.41	1.16	.16	.19	.084	0	9.0
	12 hours	7.6	4.21	.37	trace	0	0	0	4.58
Swine Manure	immediate	8.2	58.2	13.9	1.9	2.43	1.9	.53	78.86
	12 hours	7.6	44.3	76.5	.36	0	3.96	0	125.12
Domestic	immediate	6.9	33.9	74.0	.72	.24	5.55	.21	114.62
	12 hours	6.8	45.1	14.7	1.9	1.8	1.8	.47	65.77

TABLE 3. VOLATILE FATTY ACID LEVELS IN 5.1% (DRY WEIGHT) DAIRY COW MANURE

Fatty Acid	mmoles/liter[a]
acetic	43.29
propionic	6.45
isobutyric	0.31
butyric	1.36
isovaleric	0.21
valeric	0.04

a, average of three samples

TABLE 4. EFFECT OF GLUCOSE ADDITION ON LEVELS OF VOLATILE FATTY ACIDS IN A DAIRY COW MANURE DIGESTER (33 HOURS FOLLOWING ADDITION OF DAILY MANURE ± GLUCOSE)

Condition	pH	mM					
		acetic	propionic	isobutyric	butyric	isovaleric	valeric
Control	7.8	1.6	1.27	0.01	0	0	0
+ 1% Glucose	7.8	27.4	20.8	0.4	1.6	0.4	1.7
+2.9% Glucose	7.4	32.1	14.6	0.2	7.3	0.2	1.0
+4.7% Glucose	< 6	5.5	13.9	2.4	0.2	0.2	0.04
+6.5% Glucose	<6	4.0	23.2	4.1	0.4	0.3	0.2
+9% Glucose	<6	1.7	13.2	2.5	0.7	0.4	0

TABLE 5. ESTIMATED POPULATION DENSITY OF METHANOGENIC BACTERIA OF A DAIRY COW MANURE DIGESTER USING VARIOUS GROWTH MEDIA

Supplemental Additions to Basal Medium	Methanogenic Substrates	Highest Dilution in which Methane was Detected
[1]T, [2]YE, 10% [3]CMS	$CO_2 + H_2$ and other unknown compounds	10^9
T, YE	$H_2 + CO_2$	10^8
T, YE	Methanol	10^5
T, YE	Calcium acetate	10^4
None	Calcium acetate	10^6

[1] T = trypticase

[2] YE = yeast extract

[3] CMS = cow manure supernatant

TABLE 6

SULFATE CONCENTRATION OF HOT SPRING SOURCE WATERS

SPRING	MG $SO_4^{=}$/LITER
OCTOPUS	16.4
MUSHROOM	21.5
TWIN BUTTE VISTA	13.5
SERENDIPITY (WEIGERT BOARD)	12.0
WEST THUMB POOL A	26.5
BATH LAKE	700-800

Appendix A
(submitted for publication in Applied and Environmental Microbiology)

POTENTIAL INTERACTIONS BETWEEN ACETATE AND PROPIONATE TURNOVER DURING ANAEROBIC CONVERSION OF DAIRY COW MANURE TO METHANE

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Running Head -- Conversion of Dairy Cow Manure to Methane

ABSTRACT

Anaerobic conversion of dairy cow manure to methane was stable when a digester was operated with 5.1% solids at a 15 day turnover time. The low relative importance of carbon dioxide in methanogenesis (28.8%) indicated that other methane precursors (e.g., acetate) were relatively more important. Acetate conversion to methane was examined by following $^{14}CH_4$ production from [2-^{14}C]-acetate at various levels of added acetate. The reaction was saturated at about 20 mM acetate. Normal acetate levels were about half-saturating, but became saturating (27.4 mM) in response to addition of 1% glucose added to mimic increased carbon and electron flow. Glucose addition also increased propionate to 20.8 mM. Rapid conversion of [1-^{14}C]-propionate, but not [3-^{14}C]-propionate to $^{14}CO_2$ indicated that propionate decarboxylation was a major mechanism of propionate degradation. Increases in acetate, but not cations added with various acetate salts or as chloride salts, inhibited conversion of [1-^{14}C]- propionate to $^{14}CO_2$. Hydrogen also inhibited conversion of [1-^{14}C]-propionate to $^{14}CO_2$. Neither 70% hydrogen in the headspace nor 500 mM acetate completely inhibited propionate decarboxylation. Accumulation of volatile fatty acids such as propionate may be related to increased pools of hydrogen and/or acetate arising from saturation of major methane production reactions.

INTRODUCTION

Stable efficient conversion of waste organic matter to methane depends on a balance between microbial reactions involved in the production and consumption of volatile fatty acids. These two processes have in the past been considered to reflect the activity of fermenting (i.e., acid-producing) and methane-producing (i.e., acid-consuming) microorganisms. However, increased understanding of the restricted range of energy sources for methanogenic bacteria (i.e., only acetate and not other organic acids can serve as an energy source (2,7), led to the suggestion that volatile fatty acids(other than acetate)produced by fermenting bacteria are metabolized by an intermediate group of bacteria (1,2,20). Bryant (1,2) termed this group "H_2-producing acetogenic bacteria" because hydrogen and acetate would be products common to all of its members. The existence of such bacteria was difficult to imagine because of the endergonic standard free energy changes of their proposed catabolic reactions; for example:

$$\text{propionate}^- + 3H_2O \longrightarrow HCO_3^- + H^+ + \text{acetate}^- + 3H_2 \qquad \text{(reaction 1)}$$

$$\Delta G^{o\prime} = +18.2 \text{ kcal/reaction } (+76.1 \text{ kJ/reaction})$$

$$\text{butyrate}^- + 2H_2O \longrightarrow 2\ \text{acetate}^- + H^+ + 2H_2 \qquad \text{(reaction 2)}$$

$$\Delta G^{o\prime} = +11.5 \text{ kcal/reaction } (+48.1 \text{ kJ/reaction})$$

However, free energy changes are influenced by the concentrations of products and reactants (13). Recently, McInerney, et al. (8) described a bacterium obtained in coculture with H_2-consuming bacteria capable of growing by reaction 2 as well as by anaerobic oxidation of valerate, caproate and caprylate. A similar organism capable of anaerobic propionate oxidation according to reaction 1 may also exist (Boone and Smith, 1978, Abst. Ann. Meeting, Am. Soc. Microbiol., 982, p. 208; see also 2). The absolute dependence of H_2-producing acetogenic bacteria on an associated H_2-consuming microorganism attests to the requirement for product consumption in maintaining a favorable thermodynamic environment. These bacteria perhaps represent the extreme case in which interspecies H_2 transfer reactions (7,17-19) influence carbon and electron flow in natural anaerobic environments.

In anaerobic conversion of waste organic matter methane is produced by reduction of carbon dioxide (presumably via reaction 3), but mainly by reduction of the methyl carbon of acetate (reaction 4) (4,9,10), (equations from 13).

$$4H_2 + HCO_3^- + H^+ \longrightarrow CH_4 + 3H_2O \qquad \text{(reaction 3)}$$

$$\text{acetate}^- + H_2O \longrightarrow CH_4 + HCO_3^- \qquad \text{(reaction 4)}$$

In bovine waste up to about 90% of methane produced was formed from acetate (9). Under feeding conditions leading to the accumulation of volatile fatty acids in human and other animal waste conversion systems (i.e., pulse loading of organic compounds such as carbohydrate or protein polymers or monomers, increased feed concentration, decreased turnover time) acetate as well as more reduced acids (propionate, butyrate) increased simultaneously (14; Sykes, R. M., Ph.D. thesis, Purdue Univ., 1970). Because of the significance of acetate in methanogenesis in anaerobic waste conversion, the coincident accumulation of acetate and other volatile fatty acids during unstable operation, and because acetate is also produced during the oxidation of intermediate volatile fatty acids (reactions 1 and 2, for example), we were interested in the role of acetate-consuming methanogenic bacteria in the regulation of carbon and electron flow in an anaerobic waste conversion system. Experiments were performed using anaerobic dairy cow manure conversion as a model, as this material provided a stable methane fermentation over a broad range of operating conditions. Dairy cow manure is a significant source of confined waste in a rural setting such as Montana. The kinetics of conversion of acetate to methane were examined to indicate the possibility of acetate accumulation, and the effects of increased levels of acetate and hydrogen on propionate metabolism were examined.

METHODS

Primary Digester

Dairy cow waste was collected from the Montana State University dairy barn from animals which were not exposed to antibiotic treatment or to experimental changes in diet. The typical diet was approximately 30 lbs. silage, 25-40 lbs. alfalfa hay, 1.5 lbs. soybean meal, and 15-32 lbs. barley per animal per day. Fresh manure was diluted to approximately 5.1% (dry weight/liquid volume) with tap water, homogenized in a Waring commercial blender (model 31BL79), packaged in 200 ml aliquots in Whirlpack bags, and stored frozen. A primary digester was initially established at 35 C by allowing three liters of fresh manure to ferment in a four liter glass bottle for seven days. The bottle was stoppered and vented through a wet test meter (Precision Scientific Co.) so that gas production could be determined. Access to the headspace was provided by a rubber septum so that gas headspace subsamples could be removed for gas composition analysis. After active methanogenesis initiated 200 ml of fresh thawed manure was added daily after removal of 200 ml of the digester contents to achieve a 15 day turnover time. The digester under study was operated for over one year with similar characteristics.

Radiolabelling Studies

Liquid subsamples were removed from the primary digester after feeding by transferring to an Erlenmeyer flask under a stream of 70% helium/30% carbon dioxide (to prevent exposure to air and rapid reequilibration and outgassing of carbon dioxide). Small subsamples (9 ml) were then transferred via a sawed off pipette to a respirometer flask with the side arm covered by a rubber septum and the headspace was thoroughly flushed with the same gas mixture. Flasks were equilibrated for 2.5-12 hours at 37 C. In experiments using [2-^{14}C] - acetate contents of the primary digester were removed 24 hours after feeding and equilibrated for 8 hours so that the indigenous acetate level was low. Experiments were initiated by addition of radioisotopes and other compounds from anoxic solutions in distilled water by direct injection into the liquid sample through the sidearm septum. Gas headspace subsamples were periodically removed in a gas tight syringe (Hamilton) coupled to a Mininert valve (Supelco) to prevent loss of sample before analysis of CH_4, $^{14}CH_4$, CO_2 and $^{14}CO_2$. Gas production rates were monitored manometrically using a Gilson differential respirometer (model IG-14). The specific activity of carbon dioxide was determined on carbon dioxide evolved after displacement of the headspace by 100% helium so that unlabelled carbon dioxide used to gas the sample initially, did not artificially dilute the $^{14}CO_2$ evolved from added $NaH^{14}CO_3$. The specific activity of carbon dioxide was divided into the specific activity of methane (determined on a sample removed before flushing with helium) to obtain the fraction of methane derived from reduction of carbon dioxide. The time interval between collection of samples for methane and carbon dioxide specific activity determinations was less than three minutes.

Analysis of Gaseous Components

A Carle Model 8500 thermal conductivity gas chromatograph equipped with a 3.2 mm OD X 2.3 m (1/8 inch X 7.5 foot) stainless steel column packed with 80/100 mesh Poropak N (Supelco) was coupled to a Packard model 894 gas proportion analyser for analysis of CH_4, $^{14}CH_4$, CO_2 and $^{14}CO_2$. Helium carrier gas flow through the gas chromatograph was 21 ml/min. Helium make-up gas was added after combustion (at 750 C) in the gas proportion analyser to increase the total flow to 70 ml/min so that the flow of propane quench gas through the gas proportion analyser was an optimal percentage (10%) of the total flow. The gas chromatograph was operated isothermally at 50 C. Peak height from gas chromatographic data and peak area from gas proportion analyser data were compared to the responses of standards to determine gas concentrations and disintegrations per minute (based on standardization by liquid scintillation counting). Radioactivity in gases was corrected for the difference between subsample volume and the headspace volume (initial headspace volume and gas production rate times the elapsed time interval). Dpm $^{14}CO_2$ are presented as the total of $^{14}CO_2$ species in equilibrium with gaseous $^{14}CO_2$ determined after Stainton (12). In [2-^{14}C]-acetate labelling experiments the specific activity of acetate (dpm acetate added/(acetate added + indigenous acetate per vial)) was used to convert $^{14}CH_4$ to umoles CH_4 produced from acetate. Linear increases in $^{14}CH_4$ with time were noted for all vials in which acetate was added, suggesting that significant dilution of specific activity due to acetate turnover did not occur over the duration of the experiment (6 hours). In unsupplemented samples nonlinearity of $^{14}CH_4$ accumulation after 1.3 hours suggested that specific activity dilution was significant. Rate measurements for unsupplemented vials were determined from data collected within the initial 1.3 hours. In [1-^{14}C] -propionate or [3-^{14}C]-propionate labelling experiments the indigenous propionate pool was too low to obtain reliable specific activity determinations so that total dpm $^{14}CO_2$ are presented (i.e., not corrected for specific activity; see Results for controls against changes in propionate specific activity).

Analysis of Liquid Components

A pH meter (VWR pH Master) and glass combination electrode were used to measure pH. Percentage solids were determined relative to sample volume after drying at 105 C to constant weight. Volatile solids were determined by subtraction of ash weight (measured after ignition at 550 C to constant weight) from dry weight.

Volatile fatty acids were determined on samples (9 ml) clarified by centrifugation after mixing with 0.5 ml saturated aluminum sulfate, using either of the following gas chromatographic procedures:

1) On column injection in a Beckman GC-5 flame ionization gas chromatograph equipped with a 2.44 m X 3.2 mm OD (8 foot X 1/8 inch) stainless steel column packed with 80/100 mesh Chromosorb 101 (Supelco). Isothermal oven temperature was 160 C, injector temperature was 160 C, detector temperature was 160 C and helium carrier gas flow was 97 ml/min.

2) On column injection in a Varian 3700 series flame ionization gas chromatograph equipped with a 1.83 m X 2 mm ID (6 foot X 0.08 inch) glass column packed with 10% SP1200/1% H_3PO_4 on 80/100 mesh Chromosorb W AW (Supelco). Isothermal oven temperature was 115 C, injector temperature was 170 C, detector temperature was 150 C, and helium carrier flow was 40 ml/min.

Concentrations were determined by comparison of peak areas to those of standards.

Radioactivity in volatile fatty acids was determined on clarified samples (see above) using the Varian gas chromatograph system coupled via a stream splitter and a heated (150 C) teflon interface (61 cm X 3.2 mm OD, 2 foot X 1/8 inch) to the gas proportion analyser operated as described above. Peak area response was compared to that of ^{14}C-acetate and ^{14}C-propionate standards whose radioactivity was checked in 10 ml Aquasol (New England Nuclear) using a Beckman LS100-C liquid scintillation counter.

Radioisotopes

Radioisotopes were added from sterile anoxic stock solutions in distilled water. In [2-^{14}C]-acetate labelling experiments each respirometer vessel received 0.1 ml (0.5 uCi) of a 5uCi/ml stock (44 mCi/mmole, New England Nuclear). In ^{14}C-propionate labelling experiments each vessel received 1.9-3.0 uCi [1-^{14}C]-propionate (48 mCi/mmole, New England Nuclear) or [3-^{14}C] -propionate (6.3 mCi/mmole, New England Nuclear) by addition of no more than 0.2 ml (i.e., about 2% of the manure volume) from concentrated stock solutions. Maximum perturbation of indigenous volatile fatty acid pool levels would have occurred in [3-^{14}C]-propionate labelling where the added radioisotope increased the propionate pool by 15%. In NaH^{14}CO$_3$ labelling experiments each vessel received 0.1 ml (1 uCi) of a 10 uCi/ml stock (7 mCi/mmole, New England Nuclear).

RESULTS

Operating Conditions of the Primary Digester

The primary digester operated at a 15 day turnover time(5.1% solids, 86% volatile solids) provided a stable environment in which methane was produced from fermentation of organic components of the dairy cow manure. Gas production rate (50.3 ml gas/liter/hour), gas composition (53% CH_4/47% CO_2), pH (7.6) and volatile fatty acid concentrations (see Table 1) were all stable when averaged over the 24 hours between daily feeding. Variations in gas production and volatile fatty acid levels with time after feeding (see Table 1) indicated the nonconstancy of the fermentation in response to addition of fresh manure. The relative importance of CO_2 in methanogenesis was determined to be 28.8 ± 8.4% (mean of seven replicates) when specific activities of methane and carbon dioxide were compared between 2.5 and 9.5 hours following addition of fresh manure.

Response to Increased Loading

The accumulation of volatile fatty acids was examined in response to the addition of glucose (to 1% final concentration (w/v) to mimic increased flow of monomeric fermentable material in the digester. The total volatile fatty acid pool increased to more than five times the normal pool level in response to added glucose (see Table 1). The increase was due to elevated levels of all acids but predominantly to acetate (27.4 mM) and propionate (20.8 mM).

Acetate Conversion to Methane

The kinetics of conversion of $[2\text{-}^{14}C]$-acetate to $^{14}CH_4$ were examined on samples taken from the primary digester before feeding and equilibriated 8 hours to reduce the indigenous acetate pool level (0.76 mM acetate). The results

(Figure 1A) indicated that acetate conversion to methane was dependent on acetate concentration to about 20 mM. In a separate experiment the effect of added acetate on gas production rate was examined. Results (Figure 1B) indicated that gas production rate was also dependent on acetate concentration to about 20 mM. Under normal operating conditions (i.e., 4-8 mM acetate) methanogenesis from acetate appears to be near the half saturation concentration for the reaction.

Propionate Degradation

The fate of ^{14}C-labelled propionate was used to obtain empirical evidence for the mechanism of propionate degradation in the cow waste digester. Rapid evolution of $^{14}CO_2$ occurred from [1-^{14}C]-propionate but not from [3-^{14}C]-propionate (see Figure 2). The remaining radioactivity was associated with the aqueous phase and was not released after acidification. In a separate [1-^{14}C]-labelling experiment radioactivity in the acid-stable solution was shown by gas chromatography -- gas proportion analysis to be associated with propionate and no ^{14}C-acetate was detected. These results are consistent with the degradation of propionate by decarboxylation according to reaction 1. Linear increases in $^{14}CO_2$ produced from [1-^{14}C]-propionate during this and subsequent experiments (except for the indigenous condition of Figure 3) suggested that the specific activity of propionate was reasonably constant (3-4 hours maximum duration).

Effect of Acetate on Propionate Degradation

The rate of propionate decarboxylation was measured by following $^{14}CO_2$ produced from [1-^{14}C]-propionate. Increases in the acetate concentration (by addition of sodium acetate) inhibited propionate decarboxylation (Figure 3). This effect was not due to sodium or other cations added with acetate, as various salts of acetate inhibited propionate decarboxylation (Figure 4), whereas various chloride salts did not cause inhibition (Figure 5). As propionate concentration did not vary significantly during the experiments (2-4 hours) rate differences were not related to decreases in propionate specific activity.

Effect of Hydrogen on Propionate Degradation

The effect of hydrogen added to the gas headspace on conversion of [1-^{14}C]-propionate to $^{14}CO_2$ is shown in Figure 6. Increasing $^{14}CO_2$ at all added hydrogen concentrations suggested that complete inhibition of propionate degradation was not achieved. As the actual increase in dissolved hydrogen concentration depended on both diffusion and consumption, the real level of hydrogen in solution was unknown. The nonlinearity of $^{14}CO_2$ production from [1-^{14}C]-propionate in vials with added hydrogen may have been caused by hydrogen and carbon dioxide conversion to methane (reaction 3). If this was true, $^{14}CO_2$ determined from headspace subsamples would underestimate the true level of $^{14}CO_2$ produced. The decreasing headspace volume caused by consumption of hydrogen and carbon dioxide could not be quantified and was thus omitted from calculations of the total headspace volume (see Methods).

DISCUSSION

Under the operating conditions employed (i.e., 15 day turnover of 5.1% solids) dairy cow manure conversion to methane was stable as evidenced by constant gas production rate, pH and low volatile fatty acid concentrations. The relatively low contribution of carbon dioxide in methanogenesis (28.8%) suggested that other methane precursors were of greater significance. Although various compounds are known to contribute to methane production by pathways other than carbon dioxide reduction (e.g., acetate, methanol (7,16) and methyl amine (15)), acetate is considered the most significant methane precursor other than carbon dioxide in waste conversion systems, based on comparison of acetate turnover and methanogenic rate (9,10). In this respect carbon and electron flow in our system (assuming the remaining methane is produced from acetate) resembled that in other anaerobic waste conversion systems (4,9,10).

The kinetics of acetate conversion to methane were examined by following $^{14}CH_4$ production from [2-^{14}C] -acetate at various levels of added acetate. Saturation of the reaction occurred at about 20 mM acetate. Gas production also could not be increased by acetate addition above 20 mM. Under the operating conditions selected the overall conversion of waste to methane was not limited by acetate conversion to methane. In response to daily feeding the acetate pool level approached the half saturation concentration. Increased carbon and electron flow artificially induced by glucose addition led to increases in acetate levels which saturated the reaction. Concurrent propionate accumulation was noted by an increase in the ratio of propionate to acetate from 0.16 (normal) to 0.76 (after addition of 1% glucose; data of Table 1). Similar increases in acetate and propionate were observed when a cow waste digester was stressed by decreased turnover time (14; unpublished results of this laboratory), or when feed concentration was increased (14).

Because of the potential for acetate accumulation under increased carbon and electron flow to methanogenic reactions, and the coincident accumulation of propionate, we examined the effect of acetate on propionate degradation. Rapid conversion of [1-^{14}C]-propionate, but not [3-^{14}C]-propionate to $^{14}CO_2$, and the lack of production of ^{14}C-acetate from [1-^{14}C]-propionate, provided empirical evidence that propionate decarboxylation was the mechanism of propionate degradation in this digester (see reaction 1). This mechanism was also proposed by Stadtman and Barker (11) for an enrichment culture from marine sediment. The use of radiolabel provided a rapid and sensitive method for the assay of propionate degradation without substantial increase to the indigenous propionate pool. Increasing acetate levels reduced the rate of propionate decarboxylation. Significant decreases in propionate decarboxylation were observed at 10 and 25 mM acetate concentrations indicating that propionate degradation could be retarded by acetate pool levels observed during excessive loading of carbon and electrons. An increase in acetate to 500 mM, however, did not result in complete inhibition of [1-^{14}C]-propionate conversion to $^{14}CO_2$. Kaspar and Wuhrmann (5) observed complete inhibition of propionate degradation in sewage sludge when acetate was added to 80 mM, but no inhibition when acetate was added to 8 mM; however, a less sensitive method was used to measure propionate degradation.

Addition of up to 70% hydrogen to the gas headspace partially inhibited conversion of [1-^{14}C]-propionate to $^{14}CO_2$. Kaspar and Wuhrmann reported propionate accumulation (6) and complete inhibition of propionate degradation (5) when hydrogen was bubbled through digesting sewage sludge. The lack of complete inhibition by hydrogen in this study may be due to the lack of direct mixing of hydrogen with manure. Hydrogen addition stimulated $H^{14}CO_3^-$ reduction to $^{14}CH_4$ (reaction 3) in our digester (unpublished results of this laboratory). If hydrogen consumption was greater than its diffusion into solution it is possible that hydrogen addition to the headspace had only a slight effect on the dissolved hydrogen concentration. Nevertheless, our results are consistent with other results which show the negative influence of hydrogen on propionate degradation.

Kaspar and Wuhrmann (5) pointed to the requirement for low levels of both hydrogen and acetate to maintain a favorable thermodynamic environment (i.e., $\Delta G'=0$) for propionate degradation. Using data from Table 1 (0.37 mM propionate, 4.21 mM acetate), 0.2 M HCO_3^- (calculated for our digester), and $\Delta G^{o'}$ for reaction 1 (see above), the hydrogen concentration required to maintain exergonic conditions (i.e. $\Delta G'=0$) can be calculated from the following equation:

$$\Delta G' = \Delta G^{o'} + RT \ln \frac{[\text{acetate}^-][HCO_3^-][H_2]^3}{[\text{propionate}^-]}$$

where all concentrations are in moles per liter (using R=1.987 cal mole^{-1} deg^{-1}). The calculated value of 40.7 uM H_2 is probably higher than the hydrogen concentration required if ATP conservation is to occur. If we use the value used by Thauer, et al. (13) for ATP hydrolysis at physiological conditions (-10.5 kcal/mole) we calculate that 0.14 uM H_2 is required to permit ATP conservation by reaction 1. Although Kaspar and Wuhrmann (5) argued that hydrogen removal was the ecologically dominant factor, increases in either hydrogen or acetate would make reaction 1 unfavorable in our digester unless the real hydrogen concentration was lower than the calculated concentration of 0.14 uM required for energy conservation by reaction 1. Dissolved hydrogen has been reported at 1-2 uM (Strayer, et al., Abs. Ann. Meetings, Am. Soc. Microbiol., 1978, p. 166, N27) and $<10^{-4}$ atm (<0.069 uM in solution) (6) in sewage sludge, and at 0.62-1.26 uM (3) in rumen fluid. Accumulation of either hydrogen or acetate may depend on the ability of methane-producing bacteria to respond to increased production of these compounds when carbon and electron flow is stimulated (e.g., by glucose addition). In our digester acetate conversion to methane (reaction 4) was about half saturated by acetate, but became saturated on glucose addition. Although hydrogen reduction to methane (reaction 3) was not saturated, we were unable to examine the kinetics of the reaction. Kaspar and Wuhrmann (6) reported that acetate conversion to methane was closer to saturation, and thus more likely to become rate-limiting, than was hydrogen reduction of carbon dioxide to methane, in digesting sewage sludge. Although the basis for hydrogen and acetate inhibition of propionate degradation may be the generation of an unfavorable thermodynamic environment, it seems surprising that hydrogen and acetate additions did not completely inhibit propionate decarboxylation. If this reaction became endergonic by the addition of reaction products, complete inhibition would be expected.

Methane production in anaerobic waste decomposition is thought to be predominantly via the conversion of acetate to methane and the reduction of carbon dioxide by hydrogen. Acetate and hydrogen are products of the metabolism of propionate and other volatile fatty acids whose degradation depends on product removal. If production of acetate and/or hydrogen exceeds the capacity of methanogenic bacteria to consume these substrates, increasing acetate and/or hydrogen pools may cause accumulation of volatile fatty acids.

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Table 1. Volatile Fatty Acid Levels Under Various Operating Conditions of a Dairy Cow Manure Digester

Condition	mM concentration						
	acetate	propionate	isobutyrate	butyrate	isovalerate	valerate	total
Immediately after daily feeding	7.41	1.16	0.16	0.19	0.084	0	9.0
12 hours after daily feeding	4.21	0.37	trace	0	0	0	4.5
33 hours after addition of 1% glucose with daily feed	27.4	20.84	0.43	1.60	0.39	1.74	52.4

FIGURE LEGENDS

Figure 1. A. Dependence of the rate of reduction of the methyl group of acetate to methane on added sodium acetate (to achieve the mM level reported; indigenous acetate concentration was 0.76 mM). Rates were determined from linear increases in $^{14}CH_4$ from added [2-^{14}C]-acetate and the initial specific activity of acetate. See Methods for details.

B. Dependence of gas production rate on added sodium acetate during anaerobic conversion of dairy cow waste to methane and carbon dioxide. Indigenous gas production rate was subtracted so that the amount of increase due to acetate addition is shown.

Figure 2. Evolution of $^{14}CO_2$ produced from [1-^{14}C]-propionate (○) or [3-^{14}C]-propionate (●) added to an anaerobic dairy cow waste digester.

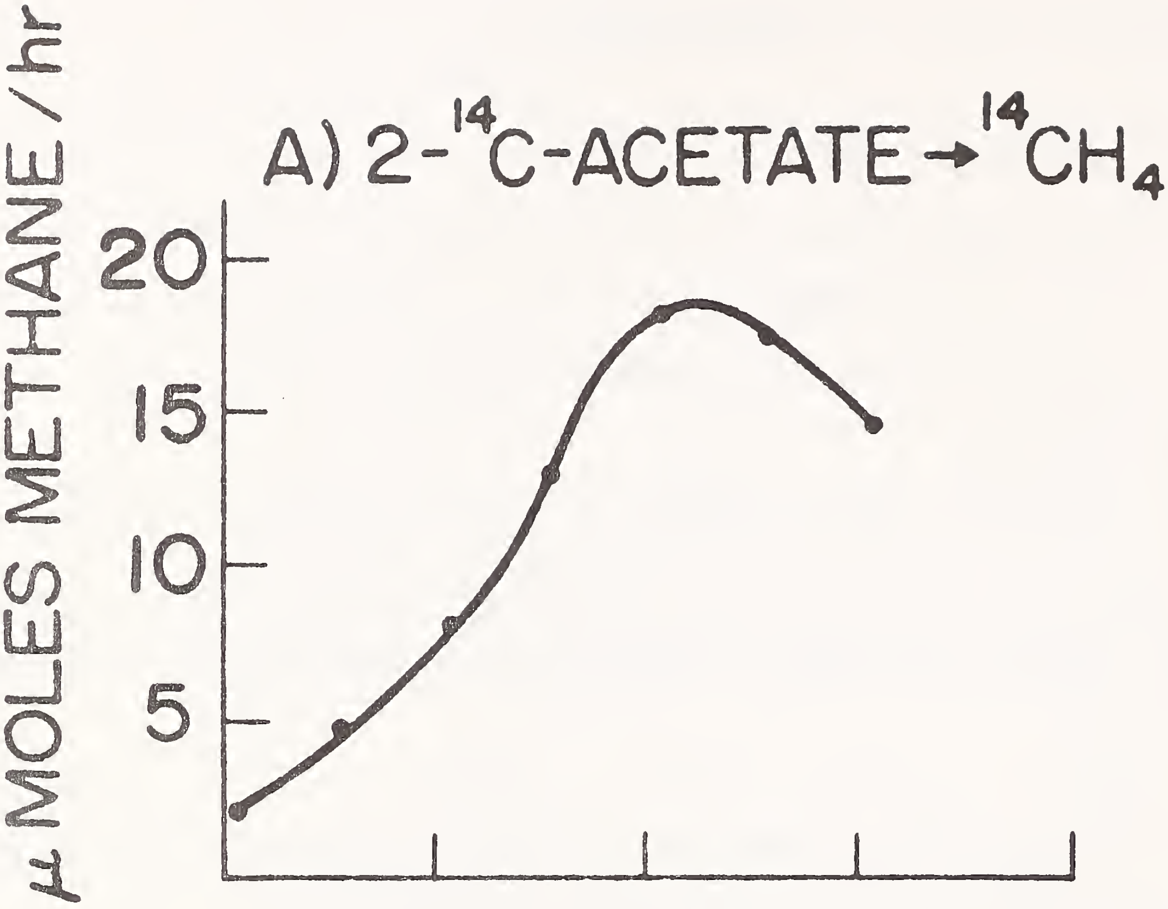
Figure 3. Effect of sodium acetate on conversion of [1-^{14}C]-propionate to $^{14}CO_2$ during anaerobic degradation of dairy cow waste.

Figure 4. Effect of various acetate salts on conversion of [1-^{14}C]-propionate to $^{14}CO_2$ during anaerobic digestion of dairy cow waste.

Figure 5. Effect of various chloride salts on conversion of [1-^{14}C]-propionate to $^{14}CO_2$ during anaerobic digestion of dairy cow waste.

Figure 6. Effect of addition of H_2 to the headspace on conversion of [1-^{14}C]-propionate to $^{14}CO_2$ during anaerobic digestion of dairy cow waste.

Fig 1


A) 2-^{14}C-ACETATE → ^{14}CH$_4$

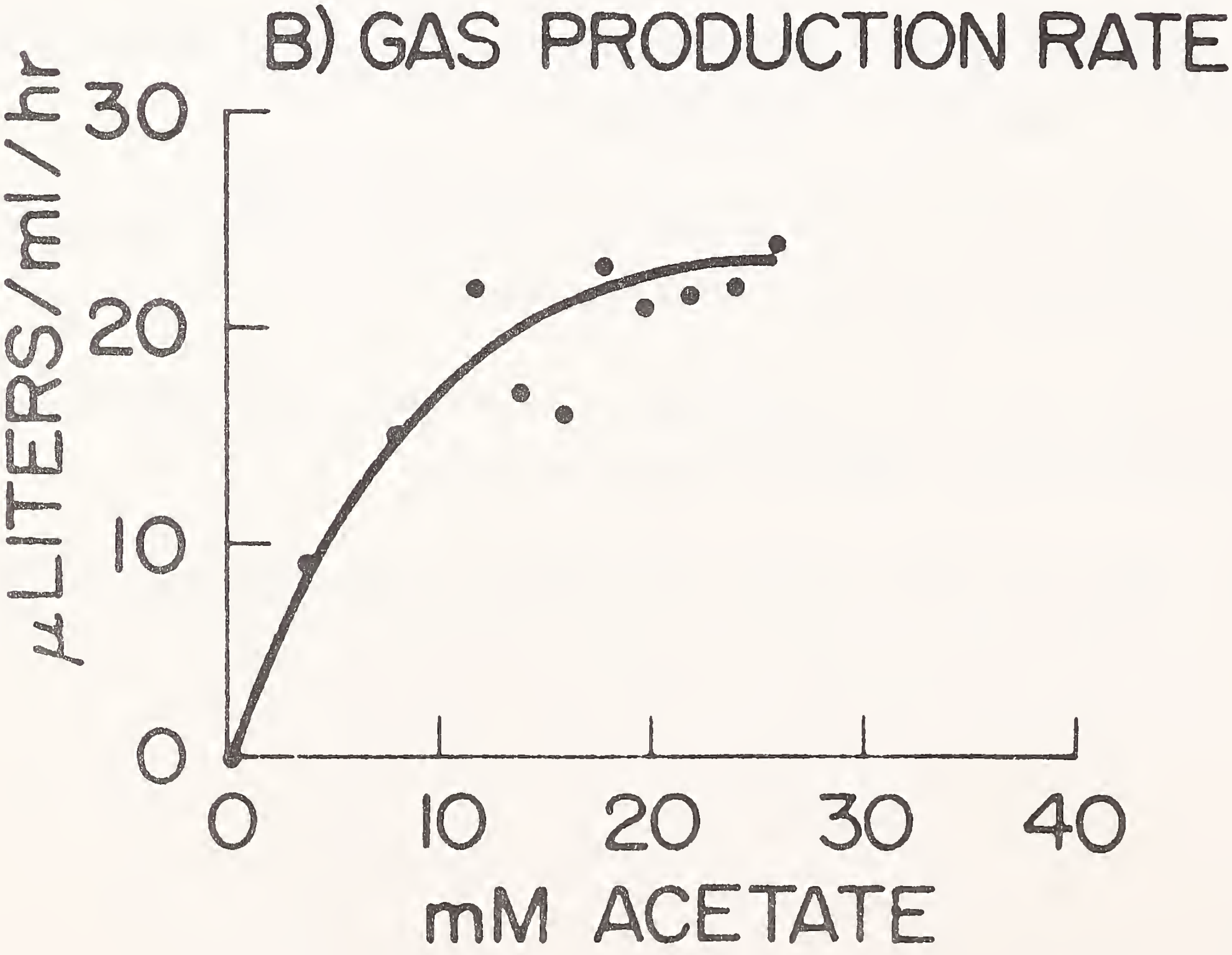
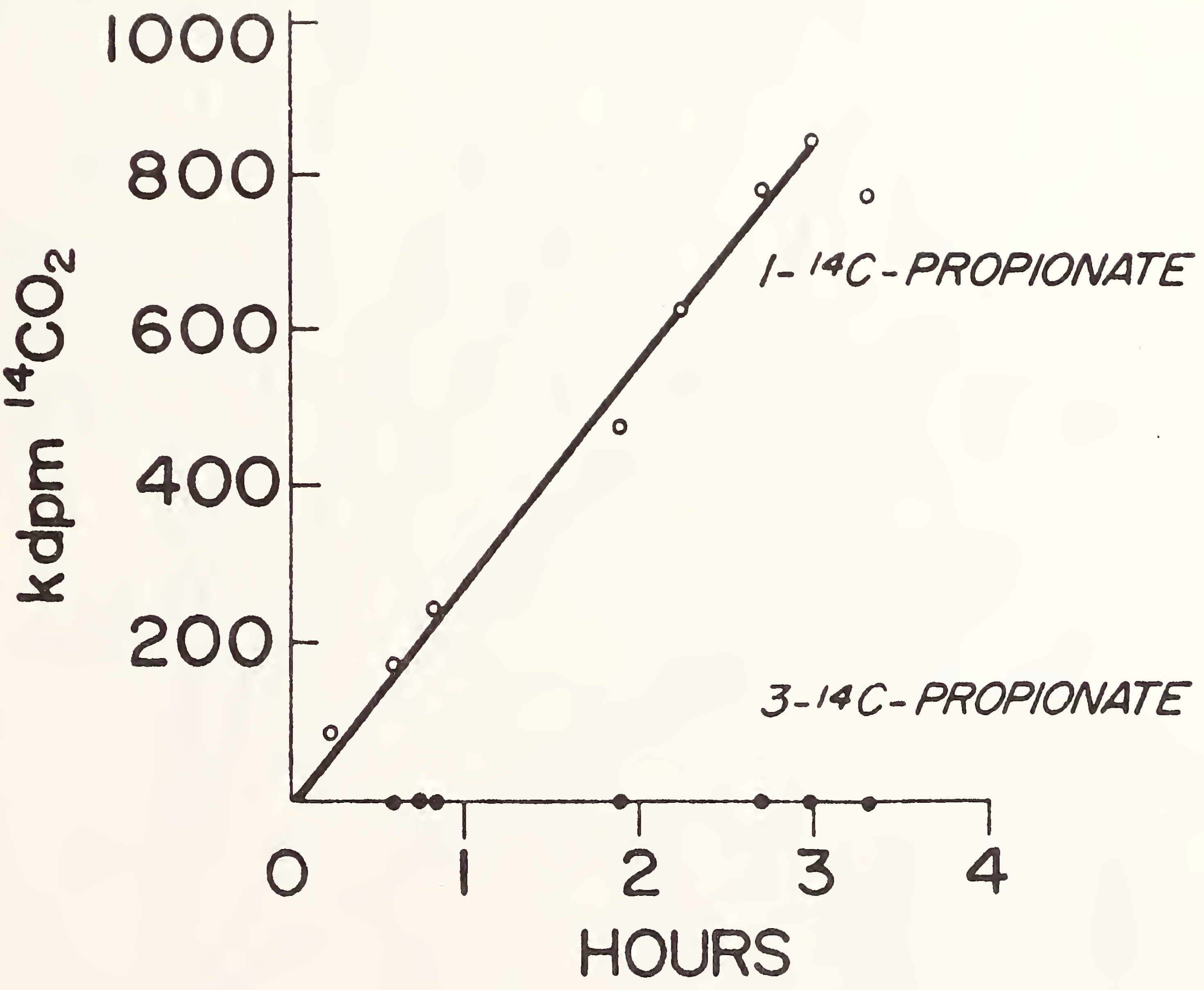

B) GAS PRODUCTION RATE

Fig 2


1000
800
600
400
200
kdpm $^{14}CO_2$
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3-^{14}C-PROPIONATE
0
1
2
3
4
HOURS

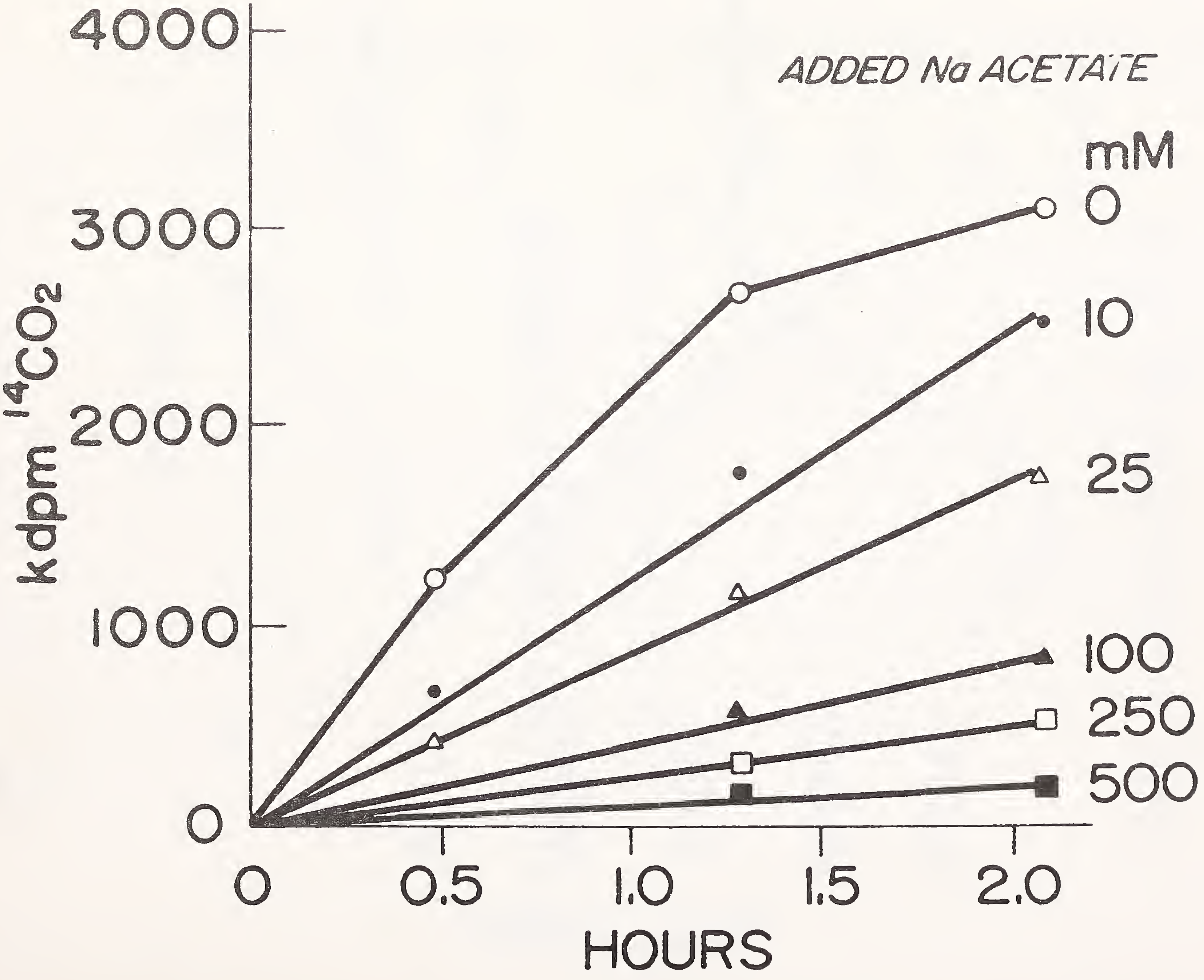

ADDED Na ACETATE
mM
0
10
25
100
250
500
4000
3000
2000
1000
0
kdpm $^{14}CO_2$
0
0.5
1.0
1.5
2.0
HOURS

Fig 4

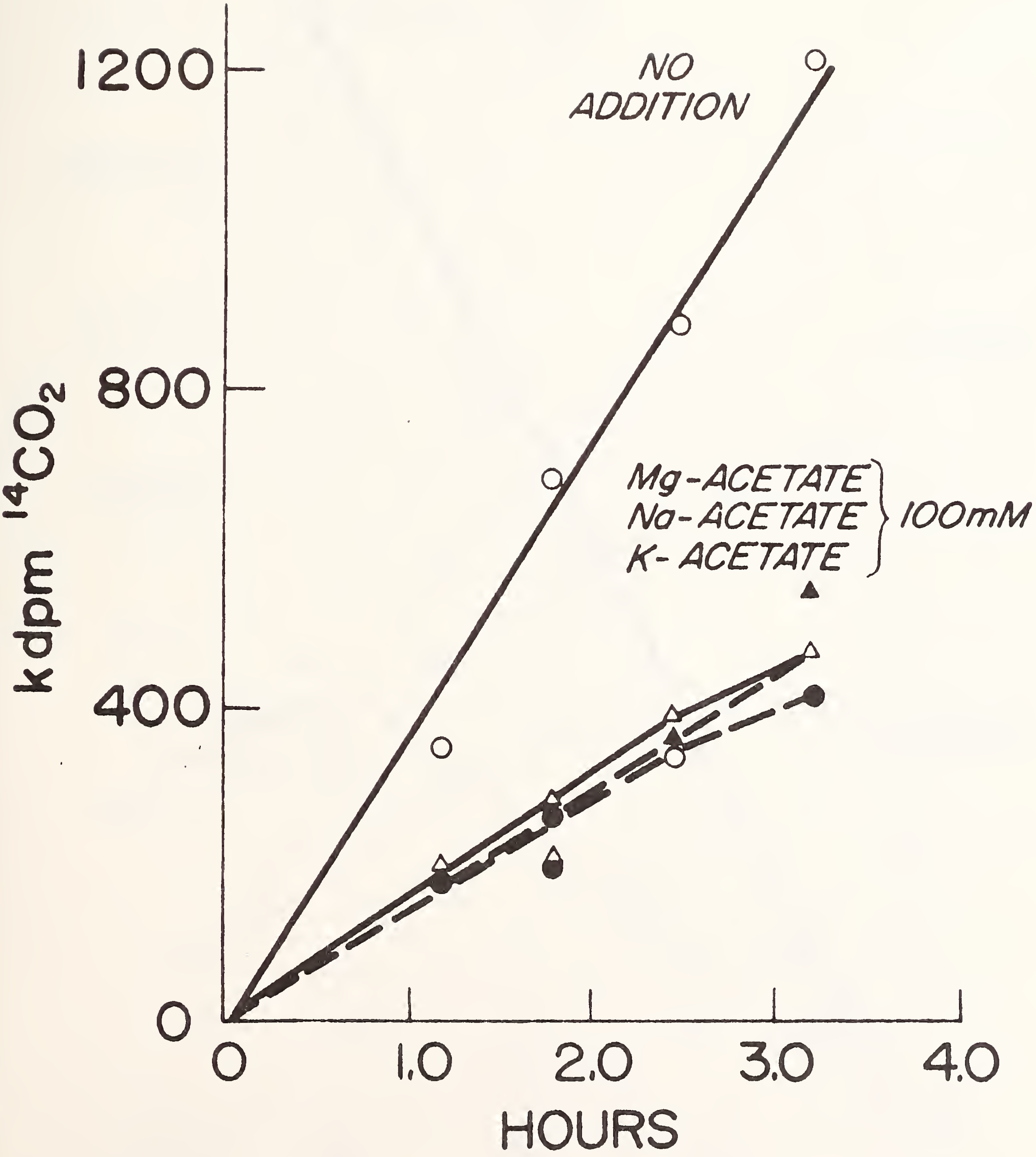
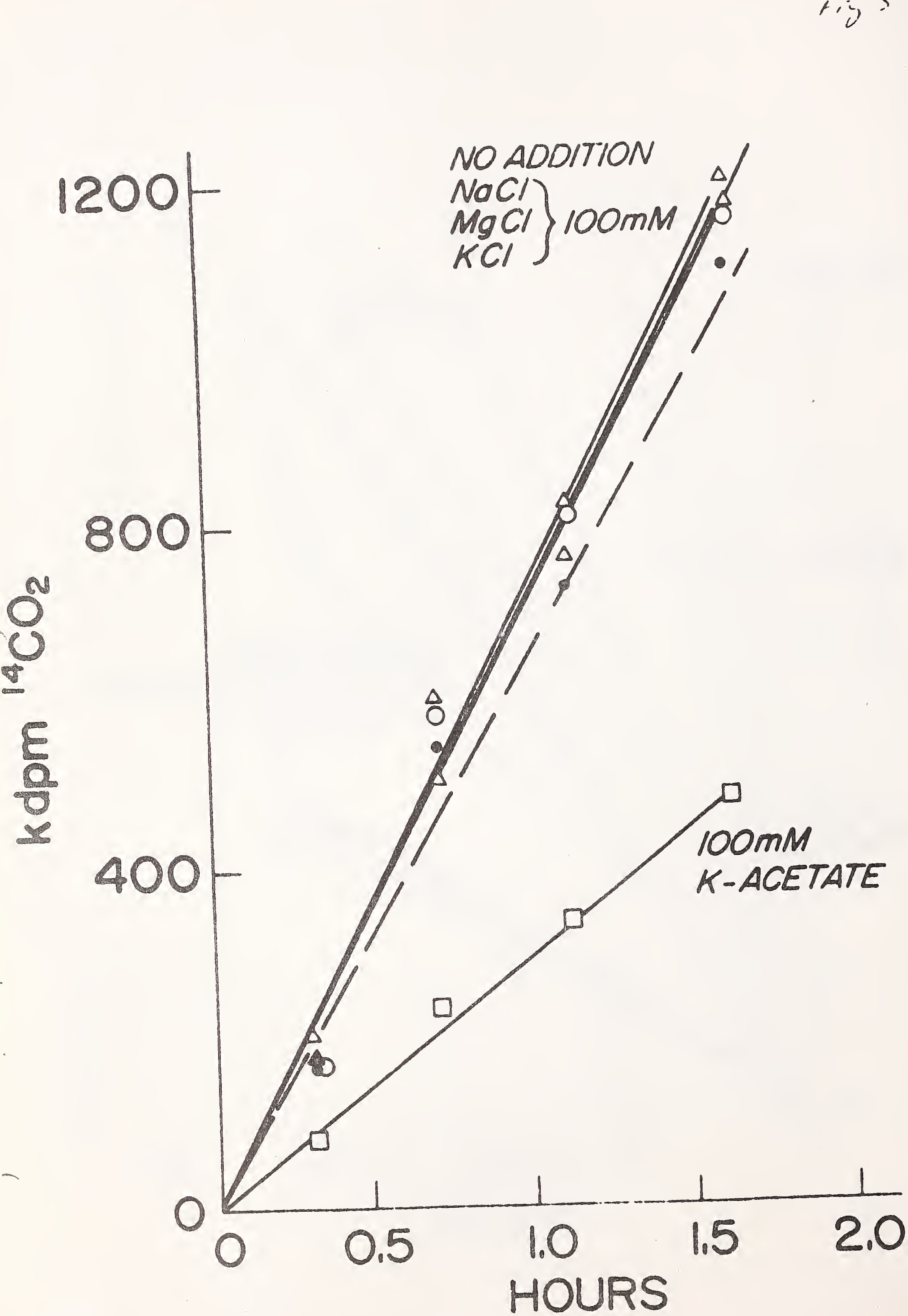
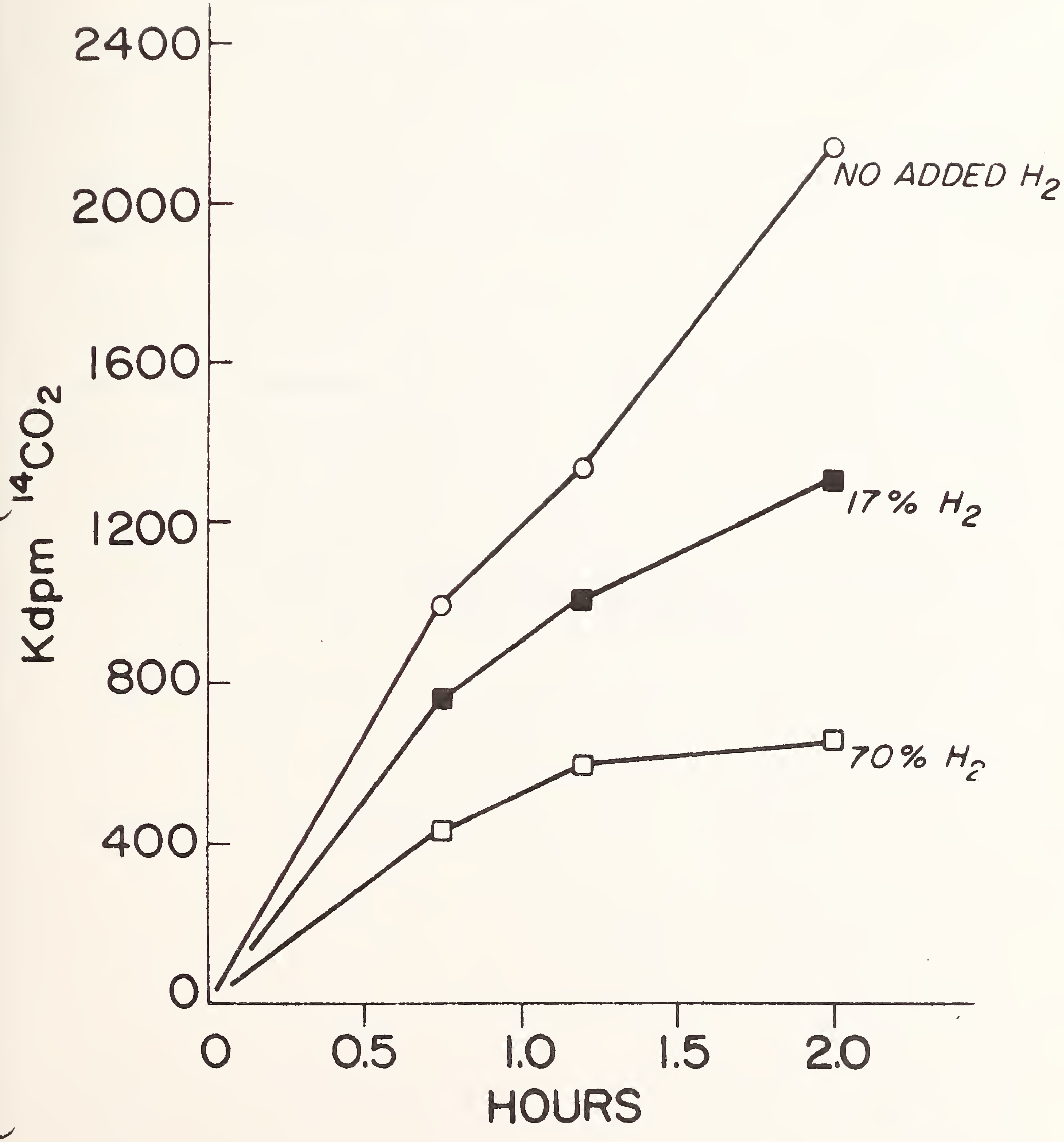

kdpm $^{14}CO_2$
1200
800
400
0
NO ADDITION
Mg-ACETATE
Na-ACETATE
K-ACETATE
100mM
0
1.0
2.0
3.0
4.0
HOURS

Fig 5



NO ADDITION
NaCl
MgCl
KCl
100mM
100mM
K-ACETATE
kdpm $^{14}CO_2$
1200
800
400
0
0
0.5
1.0
1.5
2.0
HOURS

Fig 6


2400
2000
1600
1200
800
400
0
Kdpm $^{14}CO_2$
0
0.5
1.0
1.5
2.0
HOURS
NO ADDED H_2
17% H_2
70% H_2

Appendix B
(submitted for publication in Applied and Environmental Microbiology)

TERMINAL PROCESSES IN THE ANAEROBIC DEGRADATION OF AN ALGAL-BACTERIAL MAT IN A HIGH SULFATE HOT SPRING

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Running Head - Anaerobic degradation of hot spring algal mats

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ABSTRACT

The algal-bacterial mat of a high sulfate hot spring (Bath Lake) provided an environment in which to compare terminal processes involved in anaerobic decomposition. Sulfate reduction was found to dominate over methane production as indicated by comparison of initial electron flow through the two processes, rapid conversion of [2-^{14}C]-acetate to $^{14}CO_2$ and not to $^{14}CH_4$ and the lack of rapid reduction of NaH$^{14}CO_3$ to $^{14}CH_4$. Sulfate reduction was the dominant process at all depth intervals, but a marked reduction of sulfate reduction and sulfate-reducing bacteria was observed with depth. Concurrent methanogenesis was indicated by the presence of viable methanogenic bacteria and very low but detectable rates of methane production. Marked increase in methane production was observed after sulfate depletion despite high levels of sulfide ($>$1.25 mM), indicating that methanogenesis was not inhibited by sulfide in the natural environment. Although a sulfate minimum and sulfide maximum occurred in the region of maximal sulfate reduction, the absence of sulfate depletion in interstitial water suggests that methanogenesis is always severly limited in Bath Lake sediments. Low initial methanogenesis was not due to anaerobic methane oxidation. Results are interpreted relative to results in marine and other high sulfate sediments.

INTRODUCTION

The interaction between sulfate-reducing and methanogenic bacteria has recently been described for low sulfate freshwater sediments where methane production is the predominant terminal process in the anaerobic decomposition of organic matter (3,4,5,34). In Lake Mendota sediments the inhibition of methane production by added sulfate was overcome by excess addition of electron donors for methane production--hydrogen or acetate (34). Thus, the basis for sulfate inhibition of methane production appears to be competition for common energy sources. The recent discovery of acetate-utilizing sulfate-reducing bacteria (33) provided further evidence that both methane precursors could also serve as electron donors for sulfate-reducing bacteria.

The mutual exclusion of methane production and sulfate reduction is suggested by the mutual exclusion of methane and sulfate with depth in marine sediments (see 20,22,27). Variation in methanogenesis in salt marsh sediments has also been related inversly to sulfate concentration (18). However, methane production has not been well characterized in marine or other high sulfate sediment systems (20). Although the requirement for sulfate depletion prior to methanogenesis was suggested on long term incubation of marine (21) or brackish (24) sediments, concurrent methanogenesis and sulfate reduction was reported during incubation of nearshore subtidal sediment (25). Stimulation of methane production by addition of hydrogen to nearshore sediments (25) or hydrogen and carbon dioxide in salt marsh sediment (1) suggested that competition for common electron donors may limit methane production in marine sediments. Mountfort, et al., (presented at the 4th Int. Symp. on Environmental

Biogeochemistry, Australia Acad. Sci., 1979) reported low rates of methanogenesis and predominant oxidation of [2-^{14}C]-acetate to $^{14}CO_2$ (respiratory index = $^{14}CO_2/(^{14}CO_2 + ^{14}CH_4)$ (see 35) was 0.97-0.99), indicating the predominance of sulfate reduction in sediments of Delaware Inlet, New Zealand.

In this study the terminal anaerobic processes sulfate reduction and methane production were studied in a high sulfate hot spring where an algal-bacterial mat develops at the sediment-water interface. The spring under study, Bath Lake (Yellowstone National Park), has received attention because of the sulfide-adapted thermophilic, phototrophic community comprising the algal-bacterial mat (6). This community includes the cyanobacteria Spirulina labyrinthiformis, Synechococcus lividus, Synechococcus minervae and the photosynthetic flexibacterium Chloroflexus aurantiacus (6). The anaerobic degradation of algal-bacterial organic matter to methane was shown previously in a low sulfate hot spring (32). The algal-bacterial mat of Bath Lake provided a natural high sulfate environment where organic matter was decomposed anaerobically so that the interaction between methanogenic and sulfate-reducing bacteria could be compared.

METHODS

Study Area

Bath Lake is neutral pH thermal pool formed by several springs located in the upper terrace group at Mammoth in Yellowstone National Park. The precise location was given by Castenholz (6). The sampling area was along the southern edge near sources (50-55 C) where the temperature of water overlying a uniform algal-bacterial mat was always near 40C.

Sampling and Incubation

(i) Biological Activity

Samples were removed by vertical coring with a brass cork borer (no. 4 (50.2mm^2) for whole core experiments; no. 6 (78.5mm^2) for subcore experiments) to roller culture tubes (150 x 16mm, Bellco) or two dram vials (16.75 x 60mm, Acme Vial and Glass, Co.) which were capped with recessed butyl rubber stoppers (size 00, A. H. Thomas) under a continuous flow of helium in the field (after 11). In some experiments cores were subsectioned before tubing in the field at approximate depth intervals. Anaerobically tubed samples were quickly placed in a styrofoam cooler containing warm water to keep samples within 5 C of the indigenous temperature during transit (approximately two hours). In the laboratory samples were transferred to a 40 C incubator. All additions were made from anoxic stock solutions at the time of sample collection or immediately on return to the laboratory (sulfate reduction experiments only).

The following processes were assayed as described on anoxically-tubed samples:

1. Sulfate Reduction--After addition of 0.2 ml formalin to duplicate samples (as abiological controls), replicate vials received 0.5 ml (0.2uCi) of

$Na_2{}^{35}SO_4$ (923 mCi/mmole, New England Nuclear) diluted in anoxic Bath Lake water. After 24 or 48 hours incubation replicate vials were fixed by addition of 0.5 ml 1N zinc acetate followed by 0.2 ml formalin. Counts per minute $H_2{}^{35}S$ were determined after distillation of samples acidified to less than pH 1 under N_2 into a series of three traps comprised of 0.2 ml 2N NaOH in 9 ml Aquasol (New England Nuclear). Radioactivity was trapped in the first two traps which were summed to represent total $H_2{}^{35}S$ per sample. Counts per minute added $Na_2{}^{35}SO_4$ were determined in 10 ml Aquasol. A model LS 100-C liquid scintillation counter (Beckman) was used to determine radioactivity. Sulfate was determined as described below. Correction for differences in counting efficiency were by the automatic external standard method. Sulfate reduction rates were calculated by the formula of Ivanov (12).

2. Metabolism of ^{14}C-labelled compounds--Replicate vials containing whole cores or subcores received either 0.25 ml (whole cores) or 0.5 ml (subcores) 1 uCi/ml $NaH^{14}CO_3$ (7 mCi/mmole, New England Nuclear), 0.5 ml 1 uCi/ml [2-^{14}C]-acetate (54 mCi/mmole, New England Nuclear), or 0.1 ml 33.3 uCi/ml $^{14}CH_4$ (gas, 30 mCi/mmole, New England Nuclear). Headspace subsamples were removed periodically for quantification of H_2, CH_4, $^{14}CH_4$, CO_2 and $^{14}CO_2$ as described below.

(ii) Pore Water Chemistry

Interstitial water was sampled either by squeezing water from subsections of cores (after 17), or by equilibration of a dialysis membrane sampler (after 9) filled with anoxic distilled water and incubated for 27 days in Bath Lake sediment. After removal of the dialysis membrane sampler, liquid samples were immediately removed using a helium-flushed syringe to helium-flushed butyl rubber-stoppered vials. Samples for sulfide analysis were transferred to helium-flushed vials containing 0.5 ml 2.5% (w/v) cadmium acetate in 0.2% (v/v) acetic acid to preserve sulfide until analysis. Prior to methane analysis, dissolved gases were released from 3 ml liquid samples by acidification with 0.5 ml 50% H_2SO_4. Gaseous subsamples were removed using a gas tight syringe (Hamilton) coupled to a mininert valve (Supelco) so that increased pressure did not result in loss of sample.

Analysis of Headspace Gases

Gaseous subsamples were removed from the headspace of tubes using a helium-flushed gas tight syringe (Hamilton) and analysed by gas chromatography--gas proportion analysis. For analysis of H_2, CH_4, $^{14}CH_4$, CO_2, and $^{14}CO_2$ a Carle model 8500 thermal conductivity gas chromatograph equipped with a 3.2 mm OD x 2.3 m (1/8 inch x 7.5 foot) stainless steel column packed with 80/100 mesh Poropak N (Supelco) was coupled to a Packard model 894 gas proportion analyser. Helium carrier gas flow through the gas chromatograph was 21 ml/min. Helium make-up gas was added after combustion (at 750C) in the gas proportion analyser to increase the total flow to 70 ml/min so that the flow of propane quench gas through the gas proportion analyser was an optimal percentage (10%) of the total flow. The gas chromatograph was operated isothermally at 50°C. Peak height from gas chromatographic data and peak area from gas proportion analyser data were compared to the responses of standards to determine gas concentrations and disintegrations per minute (based on standardization by liquid scintillation counting). The total amounts of H_2, CH_4 and $^{14}CH_4$ per tube were calculated by comparison of subvolume to the gas headspace volume. Total amounts of CO_2 and $^{14}CO_2$ per tube were determined by correction for the difference between subsample and headspace volume, and also for gas solubility and dissociation equilibrium according to Stainton (29). Carbon dioxide rapidly released when samples were tubed under helium was subtracted so that only CO_2 evolved during incubation is presented. Because of variation in length of cores, and the restriction of activity to the upper surface layers, results are presented per core rather than on the basis of protein content or dry weight (see also 32).

More sensitive methane analysis was performed on a Varian 3700 series flame ionization gas chromatograph using a 3.2 mm OD x 1.83 m (1/8 inch x 6 foot) stainless steel column packed with 60/80 mesh Poropak Q (Supelco) with helium carrier flow at 40 ml/min and an isothermal oven temperature of 50°C. Injector and detector temperatures were 60°C and 150°C, respectively.

Analysis of Dissolved Components

Sulfide (method 1.6 of 30) and sulfate (method 427C of 2) were determined on pore waters or on liquid samples removed after anaerobic incubations (sulfide preserved as cadmium sulfide before poisoning and analysis) by standard analytical procedures.

Volatile fatty acids were assayed by direct injection of liquid subsamples onto a Varian 3700 series gas chromatograph equipped with a 0.2 mm ID x 1.83 m (.008 inch x 6 foot) glass column packed with 10% SP 1200/1% H_3PO_4 on 80/100 mesh Chrmosorb W AW (Supelco). Isothermal oven temperature was 140°C, injector and detector temperatures were 170°C and 180°C, respectively. Helium carrier gas flow rate was 40 ml/min. Concentrations were calculated by comparison of peak area response to that of standards using a Spectra Physics model 4100 computing integrator.

Enumerations

Cores were subsectioned and tubed under helium in the field. Within a few hours, in the laboratory, subcores were suspended in prereduced liquid media prepared in roll tubes (11) designed to support growth of H_2-utilizing methane-producing bacteria (see 32, except for the use of distilled water instead of Octupus Spring water and a final pH of 7.0) or sulfate-reducing bacteria (medium E of 26). Serial dilutions were prepared by transfer in helium-flushed syringes to establish a three-tube most-probable-number assay. After 53 days incubation methane positive tubes were detected by gas chromatography as described above. After 30 days incubation sulfate reduction was recognized by the formation of a black precipitate. Further incubation did not alter the results. Most probable number was determined using standard tables (2).

RESULTS

Response of Whole Cores to Anaerobic Incubation

In initial studies it was noted that substantial methane production did not occur until after anaerobic incubation of Bath Lake algal-bacterial mat whole cores for over one week (Figure 1A). Carbon dioxide was rapidly produced after anaerobic incubation. Before the onset of significant methane production an accumulation of hydrogen was noted. In the period prior to methanogenesis the depletion of sulfate from an initially high level of 7.5 mM and hydrogen sulfide accumulation were suggestive of bacterial sulfate reduction (Figure 1B). More sensitive methane analysis suggested methane accumulation during early incubation times (Figure 2), however, the rate of methane accumulation was several orders of magnitude slower than after sulfate depletion.

During a similar incubation, accumulation of volatile fatty acids was noted after about 100 hours incubation (Figure 1C). In particular acetate and propionate levels became increasingly detectable. Although other acid levels were low, there was also evidence that butyric, isobutyric, valeric and isovaleric acids were present in higher concentration on extended incubation when compared to formalin-killed controls. Formalin also prevented accumulation of gases in the headspace and sulfate reduction (see below).

The Fate of Methane Precursors in Whole Cores

When $NaH^{14}CO_3$ was added to samples tubed anaerobically, $^{14}CH_4$ was not detected until substantial methanogenesis had initiated (Figure 3A). The addition of [2-^{14}C] -acetate at varying times after anaerobic incubation (Figure 3B) never resulted in generation of $^{14}CH_4$. When [2-^{14}C] -acetate was added at the time of anaerobic tubing or after 140 hours rapid $^{14}CO_2$ production ensued. However, neither $^{14}CO_2$ nor $^{14}CH_4$ were produced when [2-^{14}C]-acetate was added at about 260 or 430 hours (following depletion of sulfate).

Anaerobic Fate of Methane

Extended anaerobic incubation of Bath Lake whole cores with $^{14}CH_4$ did not result in generation of $^{14}CO_2$. Even after 573 hours all added label was recovered as $^{14}CH_4$.

Depth Distribution of Anaerobic Processes and Bacteria

Sulfate reduction in subcores from different depth intervals was measured by following conversion of $Na_2{}^{35}SO_4$ to $H_2{}^{35}S$ during anaerobic incubation. Sulfate reduction was markedly higher in the surface interval (approximately 5 mm) than in all deeper layers (Figure 4A). The most-probable-number of sulfate-reducing bacteria showed a similar depth distribution with an almost three order of magnitude difference between the upper ($> 10^6$ cells/subcore) and lower (10^3-10^4 cells/subcore) layers (Figure 4B). Although 95% confidence limits are omitted for simplicity a significant difference in population density is noted between the top and all deeper layers.

Methane production in subcores from various depth intervals was below detection limits during the early incubation period. Following sulfate depletion methane production increased in subcores taken from the 0-0.5, 0.5-1.0, 1.0-1.5 cm depth intervals. In deeper layers no methane production was noted on extended incubation. Methane-producing bacteria were only detected in the upper 1.5 cm depth intervals and the maximum population density was about 10^2 cells per subcore (0.5 cm interval).

A marked depth stratification was also noted for carbon dioxide production on anaerobic incubation (Figure 5). Significant increases in carbon dioxide (after rapid initial outgassing of indigenous carbon dioxide) were only noted in the uppermost depth interval.

The predominance of sulfate reduction over methanogenesis was indicated in all depth intervals by the lack of rapid reduction of $NaH^{14}CO_3$ or [2-^{14}C]-acetate to $^{14}CH_4$, and by the rapid oxidation of [2-^{14}C]-acetate to $^{14}CO_2$ (Table 1).

Interstitial Water Chemistry

Sulfate, sulfide and methane concentrations were determined after equilibration of a dialysis membrane sampler with sediment for 27 days. The results are presented in Figure 6. A similar sulfate profile was observed on analysis of pore waters removed from subcores by squeezing. Sulfate levels in the water column were between 7.3 and 8.3 mM. Although a sulfate minimum was observed in near surface layers, sulfate concentrations in deeper layers were similar to those of overlying water. A sulfide maximum was observed consistent with the sulfate minimum in near surface layers. Methane concentrations were higher within the algal-bacterial mat than in overlying water, but no consistent pattern was observed with depth in the algal-bacterial mat.

DISCUSSION

Viable populations of both sulfate-reducing and methane-producing bacteria were found to inhabit the Bath Lake algal-bacterial mat. Population densities of sulfate-reducing bacteria were from two to five orders of magnitude greater than those of the methanogenic bacteria. Very low initial rates of methanogenesis, lack of detectable $^{14}CH_4$ production from the methane precursors $H^{14}CO_3^-$ or [2-^{14}C]-acetate until over 200 hours incubation, rapid oxidation of [2-^{14}C]-acetate to $^{14}CO_2$ (consistent with the activity of acetate-oxidizing sulfate reducing bacteria (33)) and rapid reduction of $^{35}SO_4^{-2}$ to $H_2{}^{35}S$ all support the conclusion that sulfate reduction is the dominant terminal anaerobic process in the decomposition of algal-bacterial organic matter in Bath Lake. The presence of viable methanogenic bacteria and slow but detectable increases in methane during early incubation indicate that the processes concur. Oremland (25) and Winfrey and Ward (1980, in Proc. of the Int. Symp. on the Amoco Cadiz: Fates and effects of the oil spill, submitted) reported concurrent methanogenesis and sulfate reduction in nearshore subtidal and intertidal marine sediments where sulfate is also high. However, a respiratory index for [2-^{14}C]-acetate ($^{14}CO_2/(^{14}CO_2 + {}^{14}CH_4)$, (see 35) of 1.0 indicated the dominance of sulfate reduction over methanogenesis in the Bath Lake system. The data of Figures 2 and 4 were used to estimate initial rates of 0.24 nmoles CH_4 produced/core/day and 0.16 mmoles H_2S produced/liter/day (320 umoles H_2S/core/day) (assuming a 2 ml liquid volume in sulfate reduction rate experiments). Assuming that 8 electrons are required in sulfate reduction and methane production (valid only if all methane came from reduction of CO_2), electron flow through sulfate reduction was more than 1000 times greater in the initial incubation period.

The marked increase in methanogenesis upon sulfate depletion suggests that methane production is limited by the activity of sulfate-reducing bacteria. It is important to emphasize that because interstitial sulfate levels were always high, increased methane production following sulfate depletion is an artificial condition which is likely never to occur in the natural environment. The increase in methanogenesis occurred despite high levels of H_2S (>1.25 mM) further suggesting that the basis for lower initial rates of methanogenesis in Bath Lake sediment was not inhibition by H_2S as suggested by Cappenberg (3,4). The production of $^{14}CH_4$ from $H^{14}CO_3^-$ but not from [2-^{14}C]-acetate and the accumulation of acetate suggest that acetate may not be an important energy source for methanogenic bacteria in Bath Lake sediment. It is also interesting to note that acetate accumulation preceded sulfate depletion indicating a breakdown between acetate-producing and acetate-consuming reactions. This observation and the rapid uptake of [2-^{14}C] -acetate into cellular material in Bath Lake (unpublished results of this laboratory) suggest that acetate consumption by terminal members of the anaerobic food chain may not be the dominant mode of acetate utilization in this environment. The accumulation of acetate, propionate, butyrate, isobutyrate, valerate and isovalerate indicates that these compounds may be fermentation intermediates during anaerobic degradation, however, accumulation of the more reduced acids may be a result of disrupted electron flow by decreased removal of H_2 (see 20) or acetate (Kemmerling and Ward, in preparation).

The low levels of methane in sulfate-rich marine sediment zones has been interpreted as due to possible anaerobic oxidation of methane (27). In this system $^{14}CH_4$ was not converted to $^{14}CO_2$ even on incubation over 500 hours. The low rates of methane production suggest that low concentrations of dissolved methane in Bath Lake sediment are more likely due to lack of production than to anaerobic methane oxidation.

The depth stratification of the terminal anaerobic process sulfate reduction, sulfate-reducing bacteria and carbon dioxide production was striking. Similar results were found for the depth distribution of sulfate reduction (7,8,10,13,14,15,23,28,31 and Winfrey and Ward, 1980, in Proc. Int. Symp. on the Amoco Cadiz: Fates and effects of the oil spill, submitted) and sulfate-reducing bacteria (8,15,23,28) in marine (or other high sulfate) sediments and in cyanobacterial mats of a hypersaline lake (16). Interestingly, the restricted activity of methanogenesis to near surface layers in low sulfate sediments (see 20) and in a low sulfate hot spring algal-bacterial mat (32) was also noted. Methanogenic activity after sulfate depletion and methane-producing bacteria were also maximal in near surface sediment in Bath Lake. The rapid conversion of $[2-{}^{14}C]$-acetate to ${}^{14}CO_2$ and not to ${}^{14}CH_4$, and the lack of rapid reduction of $H^{14}CO_3^-$ to ${}^{14}CH_4$ in all depth intervals were further evidence of the inactivity of methanogenic bacteria in deeper layers where sulfate reduction was less active. The exclusion of methane production to deeper layers where sulfate is depleted has been suggested based on interstitial water chemistry in marine sediments (see 20). Although sulfate is not depleted by sulfate reduction in Bath Lake sediments the maximal activity of methanogenesis in near surface layers suggests that both terminal anaerobic processes are limited to upper sediment layers. Similar results were reported by Winfrey and Ward (1980, in Proc. of the Int. Symp. on the Amoco Cadiz: Fates and effects of the oil spill, submitted) for intertidal marine sediments. The basis for this stratification may be the availability of organic matter which becomes decomposed as it is buried to deeper sediment layers.

Pore water chemistry indicated a near surface sulfate minimum but not sulfate depletion. As in marine sediments sulfate depletion would be expected if ongoing sulfate reduction decreased sulfate pools. The lack of sulfate depletion may be due to replenishment of sulfate by diffusion from overlying water as the depth interval studied was not large. Another possible mechanism for replenishment of sulfate may be the reoxidation of H_2S in anoxygenic photosynthesis. Castenholz (6) reported that sulfide-adapted Spirulina labyrinthiformis from Bath Lake could use sulfide to support photosynthesis when the herbicide DCMU inhibited photosystem II. The ability of another phototroph of the mat, Chloroflexus aurantiacus, to use sulfide as an electron donor in photosynthesis has also been reported (19). In this regard maximal sulfate reduction, the sulfate minimum, the sulfide maximum and phototrophic organisms all occur in the uppermost layer of the mat.

To the extent that comparisons can be made, anaerobic degradation of algal-bacterial organic matter in Bath Lake appears to resemble organic matter decomposition in marine sediments. Carbon and electron flow is dominated by sulfate-reducing bacteria whose activity limits methanogenesis to an insignificant role in terminal anaerobic processing of organic matter. Both processes and populations are markedly reduced at depth, possibly due to lack of available organic matter. Differences from marine sediments in carbon and electron flow (i.e., acetate incorporation) and chemistry (i.e., lack of sulfate depletion with depth) may be related to the proximity of phototrophic microorganisms with the capability for photoheterotrophic growth and photosynthetic H_2S oxidation.

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Table 1. Conversion of NaH^{14}CO$_3$ to ^{14}CH$_4$ and of [2-^{14}C]-acetate to ^{14}CO$_2$ and/or ^{14}CH$_4$ during a six hour anaerobic incubation with various depth intervals of Bath Lake algal-bacterial mat cores.

DEPTH INTERVAL	NaH^{14}CO$_3$ ADDED	2-^{14}C -ACETATE ADDED	
	dpm ^{14}CH$_4$/subcore	dpm ^{14}CH$_4$/subcore	dpm ^{14}CO$_2$/subcore
0 - 7 MM	0	0	21,356
7 - 17 MM	0	0	129,514
17 - 27 MM	0	0	355,450
27 - 37 MM	0	0	475,350
37 - 47 MM	0	0	106,150
47 - 57 MM	0	0	78,637

Figure 1. Chemical changes in response to anaerobic incubation of Bath Lake whole cores.

Figure 2. Methane accumulation following anaerobic incubation of Bath Lake whole cores. Bars indicating standard deviations from the mean are included as results were near the detection limits of the method.

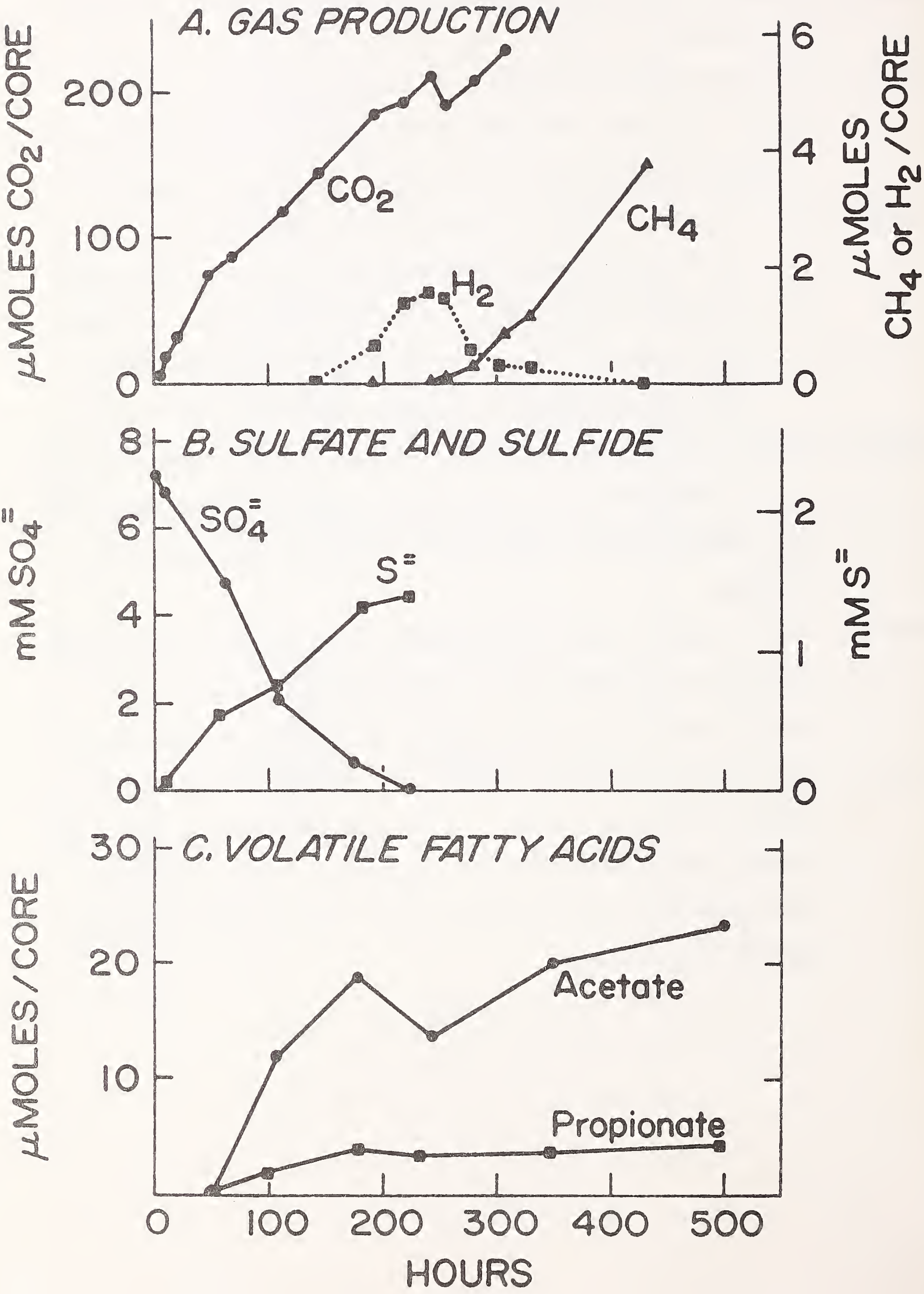
Figure 3. A. Production of $^{14}CH_4$ (dpm per core) from $NaH^{14}CO_3$ added immediately following anaerobic incubation of Bath Lake whole cores.

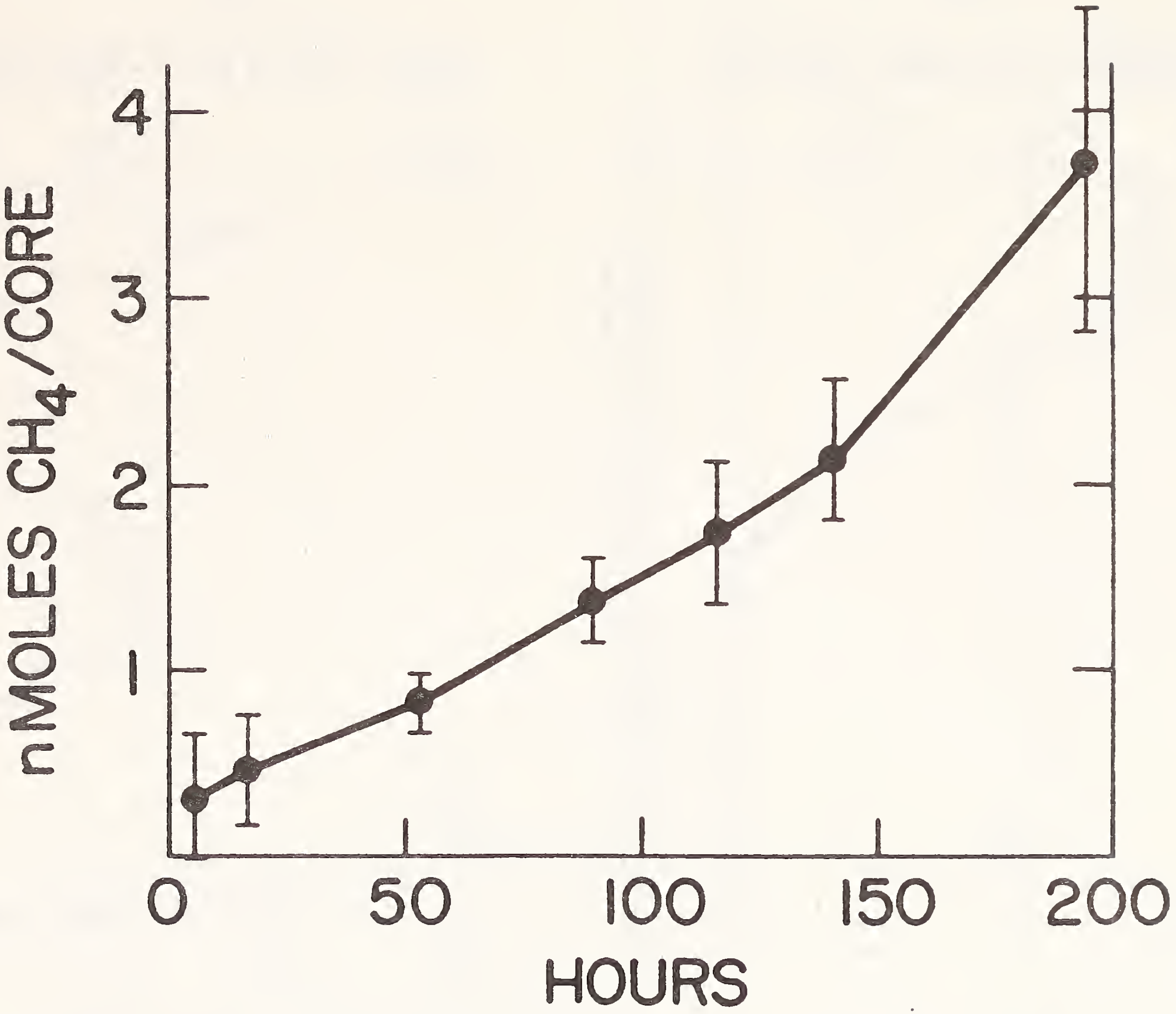
B. Production of $^{14}CO_2$ (●) or $^{14}CH_4$ (■) (dpm per core) following addition of [2-^{14}C]-acetate at various times (indicated by arrows) after anaerobic incubation of Bath Lake whole cores.

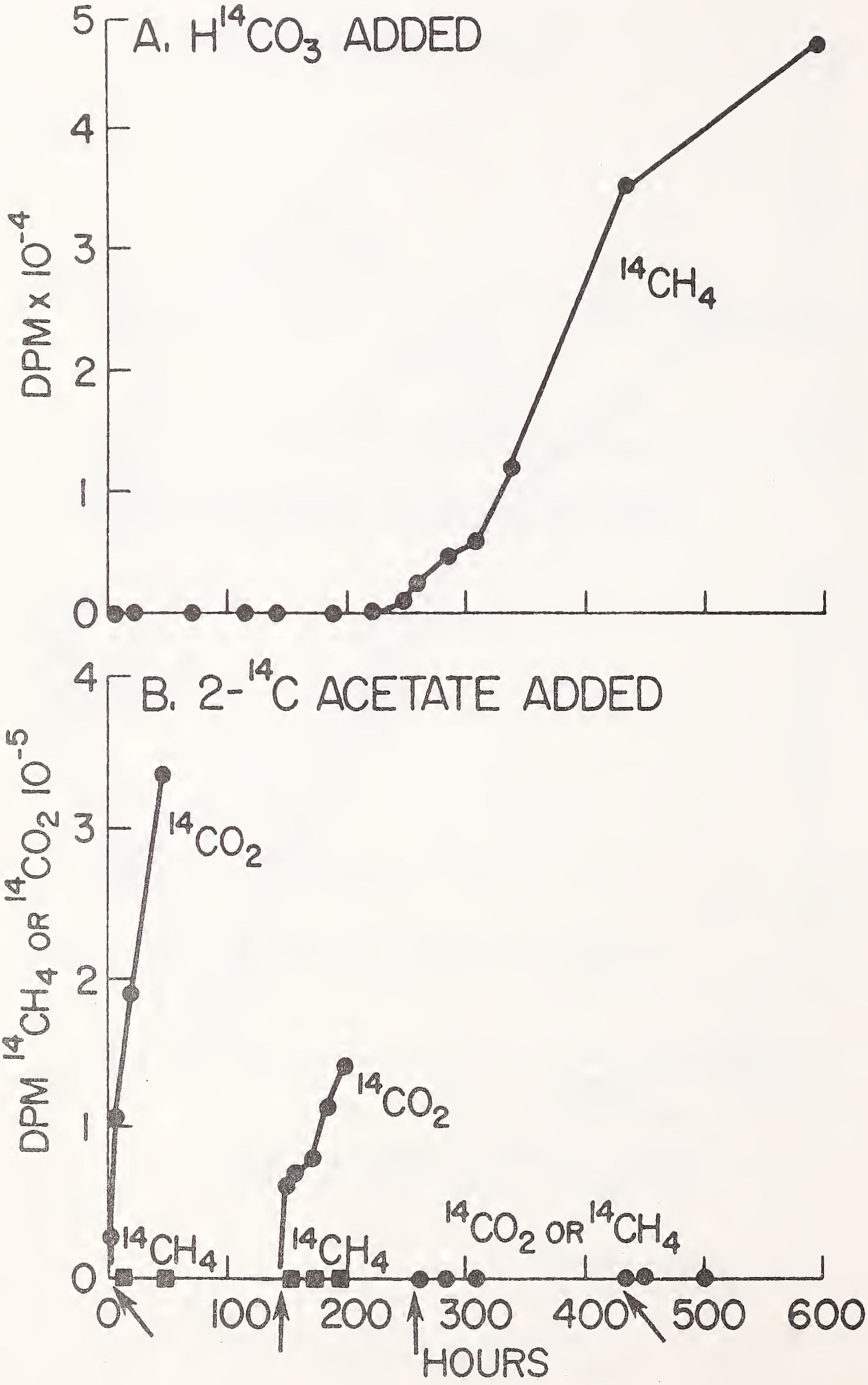
Figure 4. Sulfate reduction rate (A) and most-probable-number (MPN) of sulfate-reducing bacteria (B) in various depth intervals of Bath Lake cores. Bars indicate standard deviation from the mean of quadruplicate analyses.

Figure 5. Carbon dioxide produced during anaerobic incubation of various depth intervals of Bath Lake cores. Indigenous carbon dioxide which rapidly evolved after tubing samples under helium was subtracted.

Figure 6. Depth profiles of methane, sulfide and sulfate dissolved in pore waters of Bath Lake sediment. Dashed line indicates the interface between overlying water and the algal-bacterial mat. Pore water was sampled by 27 day equilibration of a dialysis membrane sampler with Bath Lake sediment.


A. GAS PRODUCTION
μMOLES CO_2/CORE
0
100
200
CO_2
H_2
CH_4
μMOLES CH_4 or H_2/CORE
0
2
4
6
B. SULFATE AND SULFIDE
mM $SO_4^=$
0
2
4
6
8
$SO_4^=$
$S^=$
mM $S^=$
0
1
2
C. VOLATILE FATTY ACIDS
μMOLES/CORE
10
20
30
Acetate
Propionate
0
100
200
300
400
500
HOURS


nMOLES CH_4/CORE
4
3
2
1
0
50
100
150
200
HOURS



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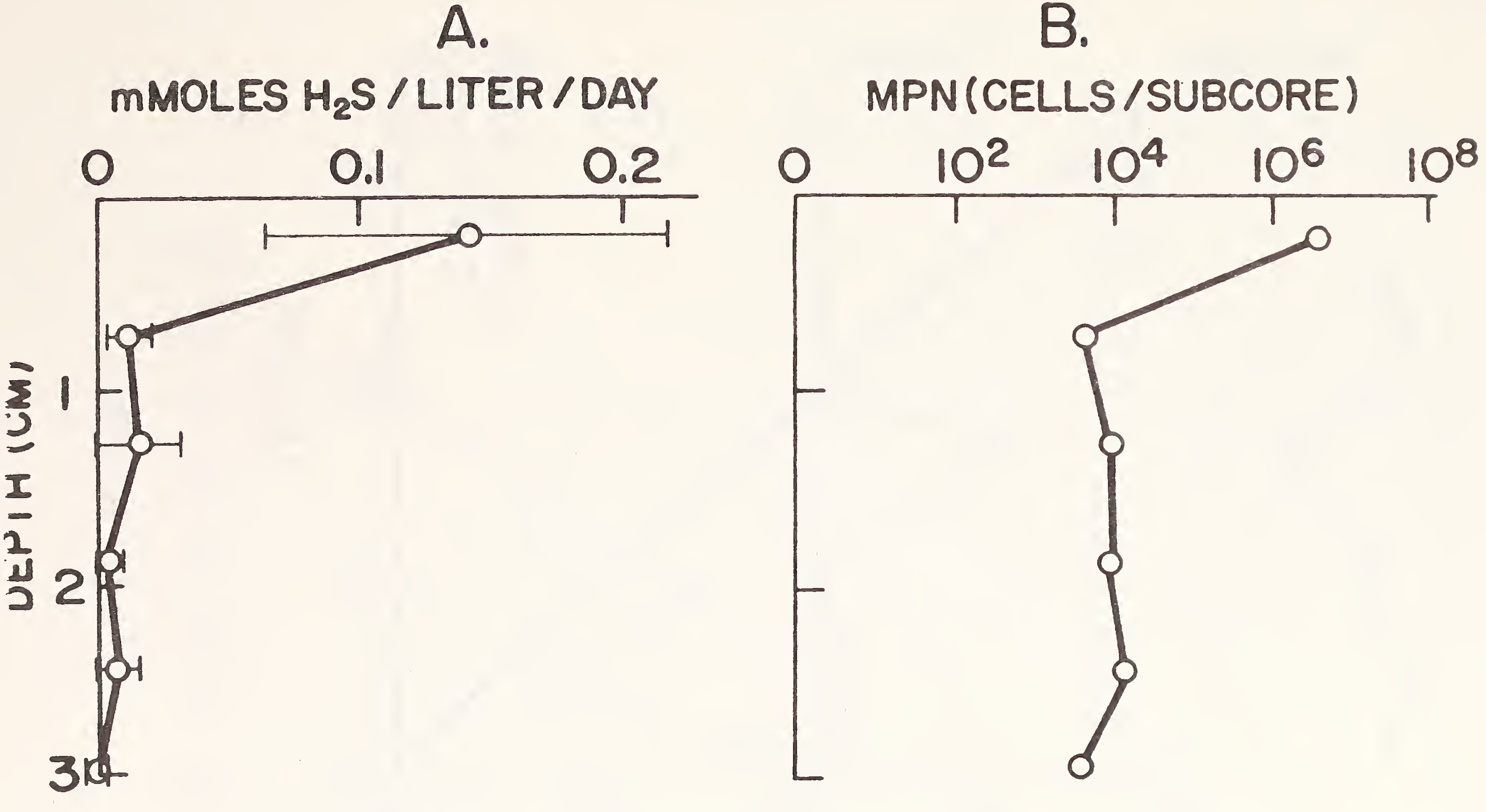
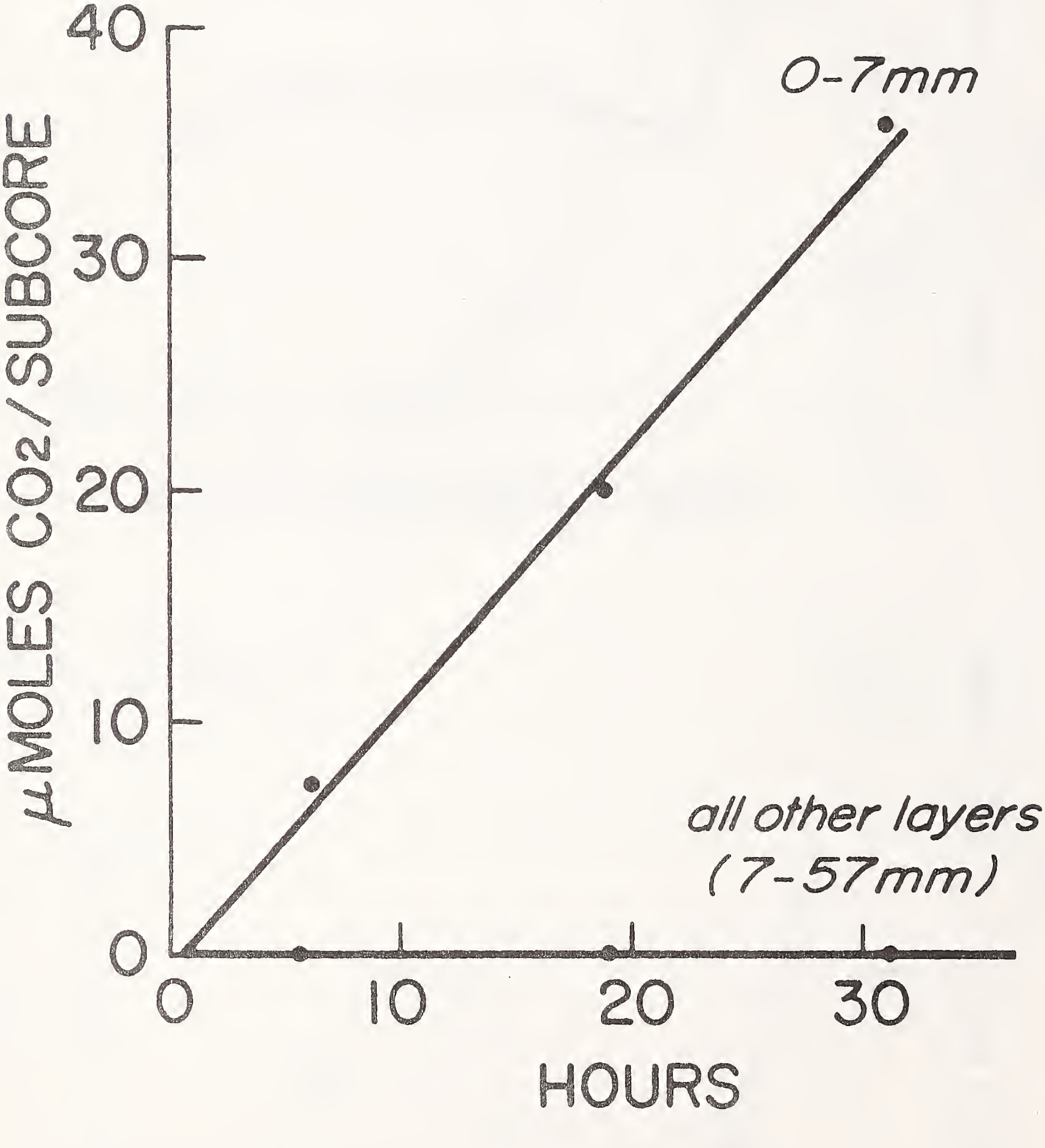
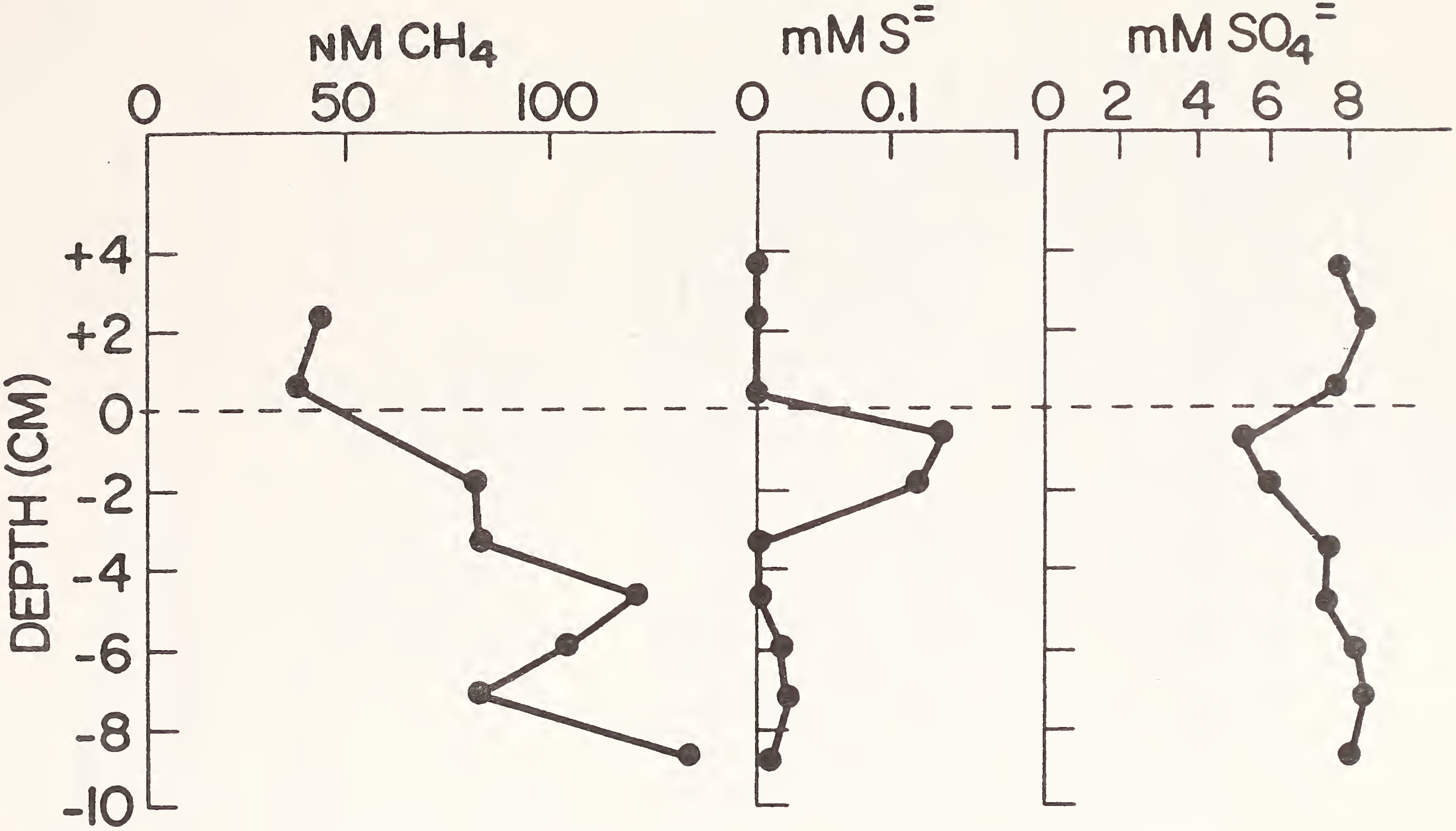
B. 2-^{14}C ACETATE ADDED


A.
mMOLES H₂S / LITER / DAY
0
0.1
0.2
DEPTH (CM)
1
2
3
B.
MPN(CELLS/SUBCORE)
0
10²
10⁴
10⁶
10⁸


40
30
20
10
0
μMOLES CO_2/SUBCORE
0-7mm
all other layers
(7-57mm)
0
10
20
30
HOURS


nM CH_4
0
50
100
mM $S^=$
0
0.1
mM $SO_4^=$
0
2
4
6
8
DEPTH (CM)
+4
+2
0
-2
-4
-6
-8
-10

Appendix C

(presented at the 1979 annual meetings of the American Society for Microbiology)

Official Abstract Form

(Read all instructions before typing)

Anaerobic Decomposition of an Algal-bacterial Mat of a High-sulfate Hot Spring. D.M. WARD* and G.J. OLSON. Montana State Univ., Bozeman, Mt.

Bath Lake is a neutral (pH 7.2) hot spring (40-56C) located in the Mammoth Terrace group in Yellowstone National Park. An algal-bacterial mat overlying the sediment consists primarily of the primary producers *Chloroflexus*, *Synechococcus*, *Spriulina*, and an organism resembling *Chromatium*. The mat and sediments provide a natural high sulfate environment where organic matter decomposes anaerobically. Pore water $SO_4^=$ was high with depth (400-800 mg/l); $S^=$ was maximal at 0.5 cm while methane was highest at depths below the $S^=$ maximum. When vertical cores were incubated under helium, $SO_4^=$ depletion and $S^=$ and CO_2 production occurred during the initial 200 hr. CH_4 production followed the temporary appearance of H_2 at 200-300 hr. 2-^{14}C-acetate was oxidized to $^{14}CO_2$ before 200 hr. $^{14}CH_4$ was produced from $NaH^{14}CO_3$ but not from 2-^{14}C-acetate after 200 hr. Vertical profiles indicated maximal CO_2 production and reduction of $^{35}SO_4^=$ to $^{35}S^=$ at 0.5 cm and maximal methane production in the adjacent lower layers--consistent with the distributions of sulfate-reducing and methanogenic bacteria. Although the presence of methane-producing bacteria and the extremely slow increases in methane indicate the concurrence of the processes, the rapid conversion of 2-^{14}C-acetate to $^{14}CO_2$ and the lack of reduction of $NaH^{14}CO_3$ to $^{14}CH_4$ at all depths indicates that sulfate reduction is the predominant process.

☐ A

☐ R

☐ RT

☐ PS

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Indicate category number from page vi	Indicate category numbers from page vi
For Los Angeles Section 53	For BOTH Los Angeles and Honolulu Section

II. JAPANESE PARTICIPANTS

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Appendix A. Conditions for rate limitations by methanogenic reactions in dairy cow manure digestions. Abstract accepted for presentation at the 2nd International Symposium on Microbiol Ecology by D. M. Ward, E. Beck and W. Rutherford.

Appendix B. Sandbeck, K. A. and D. M. Ward. Temperature relations of methanogenesis in low sulfate hot spring algal-bacterial mats. (submitted to Appl. Environ. Microbiol.)

Appendix C. Sandbeck, K. A. and D. M. Ward. Fate of immediate methane precursors in low sulfate hot spring algal-bacterial mats (Submitted to Appl. Environ. Microbiol.)

INTRODUCTION

The objectives of our research have been to understand the microbiology of methane production in two different environments. As methane is produced in totally anacobic environments and only by microorganisms, it is hoped that by understanding the fundamentals by which the microorganisms interact, improvements in the technology for waste biomass conversion to methane can be made. As the studies of the two environments represent two separate lines of research, they will be introduced and discussed separately. One of the major goals of our research has been to understand the role of methane-producing bacteria in controlling the stable, efficient operation of biomass digesters. Dairy cow manure serves as the environment in which the microbiology of waste conversion to methane has been modelled. Another major objective of our work has been to examine natural thermal environments where methane formation occurs, for the potential for thermophilic conversion of biomass to methane. Algal-bacterial mats of Yellowstone and other hot spring effluents have been the environments studied. The purpose of this report is to present technical findings and to discuss these findings relative to published literature. A nontechnical summary is also provided so that the applications of our findings can be more widely understood.

I. Animal Waste Conversion to Methane

Figure 1 summarizes the general flow of materials in anaerobic conversion of waste organic matter to methane. A complex assemblage of anaerobic microorganisms is needed to accomplish the overall process. Hydrolysis of polymers (protein, carbohydrate, fats) precedes fermentation reactions. Both reactions are probably carried out by many different anaerobic bacteria. The result of their activity is the production of volatile fatty acids (acetic, propionic, butyric, isobutyric, valeric, isovaleric acids), hydrogen and carbon dioxide. Methane is produced by a small group of anaerobic bacteria and only from a small number of compounds (Balch, et al., 1979). In waste digesters methane is produced mainly from either $H_2 + CO_2$ (Table 1, reaction 2) or acetate (Table 1, reaction 3) (Mah, et al., 1977; Zehnder, 1978; Bryant, 1979). In addition to direct production of methane precursors by fermenting bacteria, these materials are also the products of the oxidation of more reduced volatile fatty acids (propionic, butyric, valeric, isobutyric and isovaleric, but mainly propionic acid) which are also produced by fermenting bacteria but which are not directly used by the methane-producing bacteria (Bryant, 1976, 1979; Zehnder, 1978). It was suggested that these intermediate volatile fatty acids are metabolized by a group termed "obligate H_2-producing acetogenic bacteria" because both H_2 and acetic acid would be products of the proposed catabolic reactions (for example; see reaction 1 of Table 1). Because these reactions are endergonic under standard conditions the existence of such bacteria was postulated to depend on removal of products, as

$$\Delta G' = \Delta G^{o\prime} + RT\ln \frac{\text{products}}{\text{reactants}}$$

(i.e., removal of products lowers the free energy change). Recently, evidence of such a bacterium was published (McInerney, et al. (1979)). The obligate dependence of such bacteria on an associated H_2-consuming bacterium (such as a methane-producing bacterium) attests to the requirement for product removal to maintain thermodynamically-favorable conditions. In natural environments low in sulfate, hydrogen is kept in low concentration by methane-producing bacteria, as shown in reaction 2 (Table 1), so that degradation of other volatile fatty acids (mainly propionate) is more likely to proceed. However, in all natural anaerobic environments except the gastro-intestinal environment most of the methane produced comes from acetate (reaction 3, Table 1) and not from H_2 +

CO_2 (Mah, et al., 1977; see also Ivanov, et al., 1976 and Winfrey and Zeikus, 1979). In anaerobic waste conversion 70-90% of the methane produced comes from acetate (Jeris and McCarty, 1965; Smith and Mah, 1966; Mountfort and Asher, 1978).

The association between unstable operation (i.e., failure due to acid conditions) or inefficient conversion of waste to methane and the accumulation of volatile fatty acids has long been known (Zehnder, 1978). However, the basis for volatile fatty acid increases is not known. Recent theories suggest the importance of methane-producing bacteria in maintaining a balance between microorganisms which produce and consume the volatile fatty acids (Bryant, 1979; Zehnder, 1978). In the first grant period we focused on the conversion of dairy cow manure as a model system for studies of the role played by methanogenic bacteria in regulating stability and efficiency of waste conversion to methane. Under balanced operating conditions (15 day retention time of 5.1% solids waste) our system resembled other waste conversion systems in that methane was produced mainly from acetate (reaction 3, Table 1), and to a lesser extent from carbon dioxide (presumably by reaction 2, Table 1). Methane production reactions did not limit the overall conversion of waste to methane but the conversion of waste to methane was not occurring as rapidly as possible. Further evidence of stable efficient operating conditions was the maintenance of low concentrations of volatile fatty acids. Acetate, for example, was usually 4-8 mM. Acetate conversion to methane (reaction 3, Table 1) could be accelerated by addition of up to about 20 mM acetate, so that normally acetate was efficiently converted to methane but at a suboptimal rate. The probable regulatory role of methane-producing bacteria was dramatized by artificially increasing carbon and electron flow by addition of glucose (shown schematically in Figure 2). As fermentation was stimulated by addition of glucose, acetate increased to saturate the reaction in which acetate is converted to methane.

A proportionate increase in hydrogen to saturate carbon dioxide reduction to methane was also suggested by the data. Increased levels of acetate and hydrogen were shown to inhibit the main reaction catalyzed by acetogenic bacteria (reaction 1 of Table 1). Thus, increases in acetate and/or H_2 upon saturation of methane production reactions were implicated in accumulation of propionate and other volatile fatty acids. If enough glucose was added to surpass the capacity of the digester contents to buffer acid increases, pH decreased and the overall conversion of waste to methane failed. The observations of the first grant period showed the potential pivotal role played by methanogenic bacteria in regulating digester efficiency and stability.

Although stable operating conditions in the conversion of dairy cow waste were possible, the undersaturated status of methanogenic reactions indicated that the rate of waste conversion to methane was not maximal. The artificial addition of polymers (e.g., cellulose) or monomers (e.g., glucose) would be expected to stimulate methanogenesis (Robbins, et al., 1979; results of the last completion report). However, increases in carbon loading without increases in buffering capacity will eventually lead to digester failure as unbuffered volatile fatty acid accumulation decreases pH. Varel, et al., (1977) showed that increased loading of manure (by increasing either feed concentration or volumetric loading rate (this also decreases digester retention time)) led to increased methanogenesis. Maximized methanogenic rate in the stable fermentation was countered by a decreased efficiency of methane produced per amount of feed digested as volatile fatty acids accumulated. The associated volatile fatty acid accumulation was apparently buffered by increased production of ammonia upon degradation of the increased amount of waste fed so that pH did not decrease and the system was stable. From a practical point of view, studies of systems stressed by increased feeding of waste are important as they represent a natural approach to maximizing methanogenesis without pretreatment of a waste. From a basic point of view such systems have the advantage that conditions of varying efficiency (i.e., volatile fatty acid concentration) are stable with respect to time. This was a disadvantage in attempting to study the microbiological basis for volatile fatty acid accumulation by perturbation of a stable efficient system (e.g., glucose addition experiments) which led to conditions which changed with time and eventually failed. For these reasons we have concentrated in the second grant period on experimental studies of dairy cow manure digesters operated at an increased feed concentration and at various volumetric loading rates (i.e., decreased retention times). The objective remained to examine the role of methane-producing bacteria in regulating volatile fatty acid accumulation. The stability of naturally-stressed digesters led to changes in efficiency of waste conversion to methane. The causes of altered efficiency were examined and are reported here. These results have been accepted for presentation at the International Symposium on Microbial Ecology by D. M. Ward (abstract appended).

II. Methanogenesis in Natural Thermophilic Algal-Bacterial Mats

During the period covered in this report this aspect of our work was jointly funded by the National Science Foundation and MDNRC. It is hoped that an understanding of how anaerobic decomposition and methanogenesis occur in natural high temperature environments will lead to improvements in thermophilic waste conversion processes. Such natural environments are found in algal-bacterial mats which grow and decompose in the effluent channels of hot springs below about 70 C (Doemel and Brock, 1977; Ward, 1978; Ward and Olson 1980). Previous work was reported in the completion report for the initial funding period. Our work has been directed at understanding the temperature relations of bacteria involved in methanogenesis and the substrates used by methane-producing bacteria (as in Figure 1).

In contrast to what might be expected, results from the first grant period showed that methane production was optimal at temperatures well below the upper temperature limit of the algal-bacterial mats. The basis for this observation was not a lower upper temperature limit for bacteria involved in anaerobic degradation (including methane-producing bacteria) as increased temperature (to 70 C) increased methane production in samples from all temperature regions of the mat. Thus, natural thermophilic bacteria appear to be active at higher temperature than mesophilic bacteria of low temperature waste digesters, which could not be acclimated to above 60°C (Brown, et al., 1976; Cooney and Wise, 1975; Varel, et al., 1977). Some experiments reported herein address 1) the upper temperature limit for anaerobic decomposition to methane and for methane-producing bacteria, and 2) the basis for limitation of anaerobic degradation to methane to lower temperature.

Except for the primary production of algal-bacterial polymeric organic matter, the flow of materials in anaerobic degradation in this environment was initially thought to be similar to decomposition in other anaerobic environments (Figure 1). However, in the last grant period preliminary results suggested that acetate was not a major methane precursor in this system. A major direction for research during the present grant period was the study of carbon and electron flow in the terminal steps of the overall anaerobic food chain (i.e., acetate and $H_2 + CO_2$ consumption by methane-producing bacteria, as in Figure 1).

The negative influence of sulfate (and sulfate-reducing bacteria) upon methane production in anaerobic degradation of hot spring algal-bacterial mats was previously reported (Ward and Olson, 1980; appendix B of completion report to first year of funding). A third direction for research in the present grant period was the comparative study of carbon and electron flow in anaerobic decomposition between high and low sulfate hot spring algal-bacterial mats. The results of studies on high sulfate systems confirm the conclusions drawn from results of methane production in low sulfate environments.

Results of this work were presented at the 1980 annual meeting of the American Society for Microbiology and at the 1980 annual meeting of the Montana Academy of Sciences by K. Sandbeck and are now being reviewed for publication (appendices B, C).

METHODS

I. Animal Waste Conversion to Methane

Methods for initiating and maintaining dairy cow manure (see first year completion report for methods of obtaining manure) digesters are given in the text. All monitoring methods were as described in the first year completion report except for the quantification of volatile fatty acids. Manure or digester samples (2.5 ml) were clarified by mixing with 1.0 ml saturated aluminum sulfate and centrifugation (10,000 x G, 10 min, Sorval model RC2B). Clarified samples (1-2 uliter) were injected onto a 2 mm ID x 1.63 m (0.08 inch x 6 foot) glass column packed with 10% SP 1200/1% H_3PO_4 on 80/100 mesh Chromosorb W AW (Supelco) in a Varian 3700 series flame ionization gas chromatograph. Operating parameters were as follows: injector temperature, 200 C; oven temperature, 105 C for the initial 2 min followed by a programmed increase of 40 C/min to 140 C which was held for 2 min; detector temperature 180 C; helium carrier gas flow 30 ml/min. Concentrations were determined relative to recovery of hexanoic acid (used as an internal standard) and to peak area responses of standards using a Spectra Physics model 4100 computing integrator. In later analyses the internal standard was omitted due to the accumulation of hexanoic acid in one digester. Wet test meters used to measure daily gas production (see first year completion report) were calibrated with known amounts of gas before obtaining data for efficiency calculations, and the same meter was used to obtain data for all digesters.

Radiolabelling experiments were performed on 2 ml samples obtained from the primary digesters (using anaerobic technique (Hungate, 1969)) contained within 2 dram glass vials (Acme Glass Co.) and capped with no. 00 butyl rubber stoppers (A. H. Thomas) under a continuous flow of 55% helium/45% carbon dioxide (to maintain P_{CO2} at near the natural level). Experiments were initiated by addition of radiolabel and/or other compounds from sterile anoxic stock solutions in distilled water. In $NaH^{14}CO_3$ (7.5 mCi/mmole, New England Nuclear) labelling experiments, 0.2 ml (2 uCi) of a 10 uCi/ml stock was added per vial. In 2-^{14}C-acetate (44 mCi/mmole, New England Nuclear) labelling experiments, 0.3 ml (1.2 uCi) of a 4 uCi/ml stock was added per vial. In 1-^{14}C-propionate (48 mCi/mmole, New England Nuclear) labelling experiments 0.5 ml (0.5 uCi) of a 1.0 uCi/ml stock was added per vial. Hydrogen was added as 1, 2 or 3 ml 100% H_2 injected into the headspace (about 6 ml) using a glass syringe coupled to a mininert valve (Supelco). Detection of CH_4, $^{14}CH_4$, CO_2 and/or $^{14}CO_2$ in headspace subsamples was by the method described in the first year completion report, except that detector responses were integrated by a Spectra Physics model 4100 computing integrator (thermal conductivity gas chromatograph) or a Spectra Physics Minigrator (gas proportion analyser). Integrated data were treated as reported in the first year completion report to determine concentration or disintegrations per minute per vial.

Most probable number of methane-producing bacteria capable of growth on H_2 as an energy source was determined by serial dilution of digester contents in a growth medium (three tube series) similar to that used by Ward (1978) except for the substitution of tryptone for trypticase, the liquid contents of an anaerobic dairy cow manure digester for Octopus Spring water, and 80% H_2/20% CO_2 for 100% H_2 in the gas phase. The medium was made anoxic by standard anaerobic methods (Hungate, 1969). Methane production was determined after incubation at 37°C for one and two months by gas chromatographic analysis of the gas headspace as described in the last completion report. Most probable number was calculated from statistical tables (Am. Publ. Hlth. Assoc., 1976).

II. Methanogenesis in Natural Thermophilic Algal-Bacterial Mats

Study areas were described in the first year completion report (see also appendix B). Methods for these experiments were given in Ward (1978), Ward and Olson (1980), Sandbeck and Ward (appendices B, C) or in the first year completion report. In experiments performed on an Icelandic hot spring algal-bacteral mat, 0.1 ml (2 uCi) of a 20 uCi/ml stock of 2-^{14}C-acetate (44 mCi/mmole, New England Nuclear) was added per vial. Sulfate reduction rate was assayed by following the production of $H_2{}^{35}S$ from $Na_2{}^{35}SO_4$. Each vial received 0.1 ml (1 uCi) of 10 uCi/ml $Na_2{}^{35}SO_4$ (initially 738 mCi/mmole, but about two half lives expired between analysis of specific activity and use in Iceland experiments, New England Nuclear) from a sterile anoxic stock solution. Incubation was for 1 hour and 50 minutes in the effluent channel. Addition of 0.2 ml 2% Zn acetate to preserve $H_2{}^{35}S$ preceded addition of 0.2 ml formalin to terminate biological activity. In the laboratory, the sample was transferred to a glass and teflon distillation apparatus. After displacement of the gas atmosphere above the sample with nitrogen and addition of 2 ml 6N HCl to decrease pH to less than 1.0, the $H_2{}^{35}S$ was transferred (one hour under a bubbling N_2 stream) to traps consisting of 5 ml of 2% Zn acetate. After addition of 10 ml Aquasol to each trap, radioactivity was determined as described in the last completion report. The specific activity of $^{35}SO_4{}^{=}$ was determined empirically by correcting dpm $^{35}SO_4{}^{=}$ in the degassed acidified liquid (after filtration through a 0.45 um Millipore filter (counted as above in 10 ml Aquasol)) to total dpm per sample, and dividing by the total amount of sulfate in the sample (determined from sulfate analysis (method 427C, Am. Publ. Hlth. Assoc., 1976) of source water and liquid volume of the sample). The rate of sulfate reduction (nmoles H_2S/core/h) was calculated by dividing dpm $H_2{}^{35}S$ per core by the specific activity of $^{35}SO_4{}^{=}$ and by incubation time.

RESULTS AND DISCUSSION

I. Animal Waste Conversion to Methane

A. Initiation and Stabilization of 10, 5, and 3.3 Day Retention Dairy Cow Manure Digesters

Three digesters were initiated at a two liter total volume using 8.8% solids (86% volatile solids) dairy cow manure. After methanogenesis increased all digesters were fed 200 ml freshly thawed manure daily after removal of 200 ml digester contents to establish a 10 day rentention time. After a period of equilibration (based on constant gas production rate and volatile fatty acid concentration) the volumetric loading rate of two of the digesters was increased to twice and three times that of the 10 day retention digester. Thus, retention times of 5 days and 3.3 days were achieved by increasing loading rates to 400 ml and 600 ml per day. The immediate effect of increased volumetric loading was most dramatic in the digester which was switched from a 10 day to a 3.3 day retention time (Figure 3). Gas production rate and volatile fatty acid pools increased following the step up in volumetric loading. An immediate increase in the acetate pool from 2.01 mM to 20.5 mM was noted (dashed line in Figure 4). Despite metabolism of volatile fatty acids between feedings, acetate as well as propionate continued to increase over a period of days (Figure 4). An equilibrium level of acetate was achieved in which a balance between acetate production (and addition) and its consumption existed. Propionate levels had not stabilized after 8 days and eventually increased to over 60 mm (see Table 2 and Figure 13). Variations in acetate and propionate in response to daily feedings of 10 day and 5 day retention digester are shown in Figure 5. Production (and addition) of acetate or propionate initally exceeded consumption so that these acids eventually reached and maintained an average pool level which was different for each digester. All digesters eventually reached constant gas production rate and gas composition and this information and volatile fatty acid levels are summaraized in Table 2. Increased volumetric loading (i.e., decreased retention time) led to increased methane production (Table 2). Methanogenic rate in the 5 and 3.3 day retention digesters was about equivalent indicating that methane production may have been at or near saturation under these operating conditions. The methanogenic rates of these two digesters (3.24, 3.25 umoles CH_4/ml/h) were more than twice as great as that reported for a 15 day retention digester (5.1% solids) of 1.42 umoles (CH_4/ml/h (see first year completion report). Increased volumetric loading also increased levels of volatile fatty acids (mainly acetate and propionate). These increases were apparently buffered (presumably by ammonia), as pH remained constant. The concentrations presented in Table 2 are those which are removed daily. Thus these products of the overall fermentation represent an energy loss in waste conversion to methane (see below).

B. Contribution of Acetate in the Feed to Acetate Increase on Increased Volumetric Loading

One question raised in the work proposed for the present grant period was the possibility that acetate in the manure fed could contribute to saturation of acetate conversion to methane (see Figure 1). As the acetate concentration in the feed (43.3 mM, see first year completion report), and the feeding rate were known, it was possible to calculate the effect of acetate in the feed on the acetate pool immediately after feeding (Table 3). The increases predicted were in reasonable agreement with the concentration changes noted before and after feeding (10-18 mM difference for 3.3 day retention digester (Figure 4); 12 mM difference for the 5 day retention digester and 10 mM difference for the 10 day retention digester (Figure 5)). Depending on the initial pool level and the saturation status of acetate conversion to methane, the increase in acetate pool level caused by daily feed addition could lead to saturation of this methane production reaction (previous work reported in the first year completion report demonstrated that 20 mM acetate saturated reaction 3 in a 15 day retention digester).

C. Calculation of Efficiency of Waste Conversion

The efficiency of waste conversion was calculated according to the method of Varel, et al., (1977), based on the assumption that the organic matter decomposed was 40% carbon. In this calculation the carbon recovered in the gaseous end products methane and carbon dioxide is compared to the combustible carbon (volatile solids) fed so that a percentage conversion to these products is determined. As all combustible carbon is not amenable to anaerobic biodegradation the calculated conversion efficiency is usually much lower than 100%. Robbins, et al. (1979) reported conversion efficiencies using this calculation method of 30-35% for 16 day retention digesters of 5, 6 and 7% manure concentrations, using the same manure source as in our experiments. Thus, increases in feed concentration to 7% at a 16 day retention time did not affect the efficiency of waste conversion to methane. The efficiency of waste conversion to methane for the 10 day retention digester was 34.3% indicating that the digester was not stressed by more rapid turnover at an even higher feed concentration (Table 4). As the digesters were shifted to shorter retentions, resulting in increased methanogenic rate and volatile fatty and concentrations, the efficiency of waste conversion to methane decreased to 28.1% in the 5 day retention digester and to 19.6% in the 3.3 day retention digester (Table 4). In practical terms, increasing the feeding rate of a digester led to a 3.35 times greater methanogenic rate from 0.97 µmoles/ml/hr. (calculated from data of Robbins, et al., 1979 for 16 day retention of 7% volatile solids waste) to 3.24 µmoles/ml/h (5 day retention digester) with only a slight reduction in conversion efficiency. This was accomplished merely by accelerating the rate at which the digester was fed with untreated dairy cow manure. Further increase in feeding rate (3.3 day retention) did not improve methanogenic rate but led to inefficiency as waste conversion to gases was about half as efficient as in digesters which were fed at slower rates.

Associated with the inefficient conversion of wastes to gaseous products was a large increase in volatile fatty acids. Because of the large buffering capacity of dairy cow manure, the accumulated acids did not cause a lowering of pH. The resulting digester was stable but inefficient, providing an environment in which to study the microbiological basis for volatile fatty acid accumulation without rapidly changing conditions followed by digester failure. This was a problem in experiments in which a stable efficient digester was artificially made unstable (see first year completion report). The efficiency of waste conversion to all products (i.e., CH_4, CO_2 plus volatile fatty acids) was constant among the three digesters (Table 4, standard deviations overlap indicating that there is not a significant difference among the data for different digesters). This means that a doubling and tripling of the feeding rate resulted in a doubling and tripling of the amount of waste broken down. Apparently the hydrolysis and fermentation reactions which preceed methanogenic reactions (see Figure 1) were not operating at maximal rates , even in the 3.3 day retention digester, as their activity was stimulated by addition of more polymeric organic matter in the waste. Although the 3.3 day retention digester was inefficient in waste conversion to methane, it was efficient in waste conversion to methane plus volatile fatty acids (mainly propionate and acetate). This may have practical advantage if volatile fatty acids are useful as a product (e.g., as chemical feedstocks, as animal feed). The carbon and electrons recovered as volatile fatty acids were comparable to the carbon and electrons which would have been present as methane and carbon dioxide had the efficiency of waste conversion to gases in the 5 and 3.3 day retention digester been constant (Table 5). The highest feeding rate resulted in no further increase in methanogenic rate, and to a stoichiometric diversion of carbon and electrons from the products methane and carbon dioxide to volatile fatty acid products, indicating the probable saturation of methane production reactions.

D. Status of Methane Production Reactions

Indigenous methanogenic rates measured in small samples of contents of each digester (Figures 6 and 7) compared well to the average methanogenic rate determined from the daily gas production rate times % CH_4 (Table 2). The 3.3 and 5 day retention digesters had greater methanogenic rates than did the 10 day retention digester but the difference between methanogenic rate in the 3.3 and 5 day retention digesters was insignificant (Table 2 and Figures 6 and 7). Increased volumetric loading led to increased acetate (and presumably hydrogen) concentrations (Table 2). As predicted by the concentration dependence of acetate conversion to methane (presented in the first year completion report) methane production reactions should have been increasingly saturated upon increased volumetric loading. The degree of saturation of methane production reactions was assessed in the various digesters by examining the effect of addition of varying amounts of sodium acetate or hydrogen on methanogenic rate. In the 10 day retention digester all concentrations of added acetate and hydrogen increased the rate of methane production (Figures 6 and 7), indicating that both reactions were undersaturated. In the 5 day retention digester all concentrations of added acetate or hydrogen had about the same stimulatory effect on methanogenic rate and the degree of stimulation was less. Both methane production reactions are apparently close to saturation in this digester. Increases in acetate or hydrogen had no effect on the rate of methane production in the 3.3 day retention digester, indicating that both methane production reactions were saturated by the high levels of acetate (and presumably hydrogen) in this digester. These findings agree with earlier results in a 15 day retention digester which suggested that acetate conversion to methane became saturated at about 20 mM. Only in the 3.3 day retention digester was acetate near the level required to saturate the reaction. Although hydrogen pool levels were not determined, and the dependence of reaction 2 on hydrogen concentration is unknown, the results argue that this methane production reaction was also saturated in the 3.3 day retention digester.

The influence of hydrogen on reaction 2 was studied more precisely by observing conversion of $NaH^{14}CO_3$ to $^{14}CH_4$ at various levels of added hydrogen in the 10 day retention digester. The results presented in Figure 8, also indicated the reaction was undersaturated -- similar to the effect of hydrogen on methanogenic rate (Figure 7). A relative importance of 10-24% for reaction 2 in methane production (determined by comparing specific activities of $^{14}CH_4$ and $^{14}CO_2$, see appendix C for discussion of this method) in the 10 day retention digester (Table 6) was similar to that reported for a 15 day retention digester (see first year completion report). Added hydrogen increased the relative importance of reaction 2 to 41-58% as indicated by lower isotopic dilution in the presence of hydrogen (Table 6).

These results confirm the suggestion that methane production reactions become rate-limiting for the overall conversion of wastes to methane in the 3.3 day retention digester, as further increases in methanogenic substrates did not stimulate methanogenesis.

E. The Problem of Population Washout

The accumulation of methane precursors (e.g., acetate, H_2) or reduced volatile fatty acids (e.g., propionate) could be a result of the lack of sufficient bacterial populations which catalyze their degradation (e.g., H_2- or acetate-using methanogens, propionate-degrading acetogens). This is a potential problem in our experimental approach as increased volumetric loading is accomplished by shortening the retention time of materials in the digesters. Obviously, if bacteria do not grow as fast as they are washed out by the passage of manure through the digester, their population density will be decreased. Methane-producing bacteria are known to grow in pure culture at rates sufficient to prevent population reductions in the 3.3 day retention time digester (see Smith and Mah, 1980). The evidence presented above (and in section I.G.2, p. 20) suggests that both methane production reactions are normal in this digester.

Propionate-degrading acetogenic bacteria which have been described in coculture (because they cannot be grown in the absence of bacteria which consume their products) have not been found to grow at rates sufficient to prevent dilution in 5 day or 3.3 day retention digesters (Boone and Bryant, 1980). The hypothetical changes in acetogenic bacterial populations based on maximum reported growth rates and 5 day and 3.3 day retention times are shown in Figure 9. It was necessary to perform experiments which showed whether or not acetogenic bacteria capable of degrading reduced volatile fatty acids were present in the 3.3 day turnover digester. Because of the extreme dependence of acetogenic bacteria on product removal, and the dissimilarities of cultural and natural environments, it is likely that these bacteria could grow at faster rates in natural environments than in culture. The oxidation of 1-^{14}C-propionate to $^{14}CO_2$ was used to assay the presence of propionate-degrading acetogenic bacteria (Figure 10). The rate of propionate oxidation was similar in all digesters suggesting that population densities of propionate-degrading bacteria must have been relatively similar. The experiment was performed nearly three months after the 3.3 day retention digester was initiated. At the reported maximal growth rate the population would have been diluted by more than seven orders of magnitude (Figure 9). The results imply that a significant propionate-degrading acetogenic population was present and able to grow at a rate faster than maximum reported growth rates in culture. The lack of propionate-degrading bacteria was not apparently the basis for propionate accumulation in the stressed digesters.

F. The Basis for Propionate Accumulation

The accumulation of propionate and other volatile fatty acids must be due to an imbalance between its production and decomposition. Because of the sensitivity of acetogenic bacteria to product (i.e., H_2 (see Bryant, 1976, 1979; McInerney, et. al., 1979) and acetate (Kaspar and Wuhrmann, 1978; see last completion report)) accumulation, we were concerned that the increased levels of acetate (and presumably H_2) in the digesters with shorter retention times could inhibit or at least decrease propionate degradation. However, rates of propionate oxidation were approximately equivalent in all digesters (Figure 10) indicating that thermodynamic conditions were favorable for propionate degradation in all digesters. The tolerance of the reaction (reaction 1 of Table 1) to hydrogen concentration could be calculated as the concentrations of all other substrates and products in the three digesters were known (see Table 7). The elevated levels of the substrate propionate in 5 day and 3.3 day retention digesters counteracts the elevated acetate level so that the stressed digesters actually become more tolerant to hydrogen. Hydrogen was apparently present at lower levels than presented in Table 7, as propionate degradation was occurring, indicating thermodynamically feasible conditions for the reaction. It must be noted, however, that this experiment was performed more than 3 months after the initiation of the 3.3 day retention digester. Results presented below suggested that long term changes in the digester may have significantly lowered the H_2 concentration by the time of the experiment.

Thus, the basis for the initial accumulation of propionate could have been related to H_2 and/or acetate inhibition of propionate oxidation.

The basis for propionate accumulation, and thus digester inefficiency and instability, remains a major objective for future research. It is hypothesized that an increase in propionate formation could result in propionate accumulation. This could occur as a result of accumulation of H_2 as its consumption by methane-producing bacteria reaches its maximal rate. Preliminary experiments have been performed to develop methods to study the flow of fermentation products toward reduced volatile fatty acids (e.g., propionate) and away from methane precursors (e.g., acetate) as methanogenic reactions become saturated. Additional experiments on the rate of propionate oxidation during the period of propionate accumulation must also be performed.

G. Acclimation of Microbial Populations

The inefficient conversion of waste to methane at short retention time appears to be related to saturation of methanogenic bacteria with their energy sources. It is logical to expect microbial populations in a continuous flow environment to respond to increased fluxes of energy sources by increasing population density if the supply of energy source limits growth. A resulting decrease in volatile fatty acids and increase in methanogenic rate and efficiency in waste conversion to methane would be predicted. Several experiments have been performed to study long term changes in the various digesters. These experiments are also needed to ensure the long range pH stability and conversion efficiency of digesters operated to maximize methanogenesis, so that scale up applications would not fail due to insufficient base line knowledge of the systems' stabilities over long time periods.

1. Changes in population density of methanogenic bacteria.

Four and one-half months following the initiation of the 5 and 3.3 day retention digesters, an enumeration experiment was performed to examine whether methanogenic bacteria increased in response to increased supply of energy sources. Most probable numbers of H_2-utilizing, methane-producing bacteria were 4.3×10^8, 1.5×10^9, and 4.3×10^9 cells/ml, in the 10 day, 5 day and 3.3 day retention digesters, respectively. The results suggest that these bacteria had increased in the digesters operated at shorter retention time. As no change in the relative importance of methanogenic reactions was noted (see below) it is likely that acetate-using methanogenic bacteria also increased in population density. However, experiments on the relative importance of methanogenic reactions were performed nearly two months before the enumeration experiment.

2. Changes in the saturation of methane production reactions.

An experiment on the effect of H_2 additions on reduction of $NaH^{14}CO_3$ to $^{14}CH_4$ was performed three months after initiation of the 3.3 day turnover digesters. The results (Figure 11) indicated that this methanogenic reaction was not saturated. This was in contrast to the observation that H_2 did not stimulate methanogenesis in experiments performed one month after initiation of the 3.3 day retention digester (Figure 7), and suggested that the increase in the population of H_2-utilizing methanogens may have reduced the H_2 concentration to a level which did not saturate methanogenesis. Specific activity comparisons indicated that the relative contributions of methane production reactions were similar to those in the 10 day retention digester (Table 6). Thus, both populations of methane-producing bacteria may have acclimated to increases in H_2 and acetate. This was also suggested by the observation that addition of acetate stimulated reduction of 2-^{14}C-acetate to $^{14}CH_4$ (Figure 12) in an experiment performed about four months after initiation of the 3.3 day retention digester. This was in contrast to the saturated status of acetate conversion to methane observed about one month after initiation of the digester

(Figure 6). The results suggested the possible acclimation of methane-producing bacteria over a period of several months, and were consistent with the increased population density of methanogenic bacteria.

3. Changes in volatile fatty acids and stability.

Following the initial increases in volatile fatty acids which occurred after initiation of the 3.3 day retention digester, volatile fatty acid consumption eventually equaled production, as indicated by the lack of further increase in acetate and propionate (Figure 13). During the next 3-4 months acetate and propionate concentrations slowly decreased. This could have resulted from changes in the density of microbial populations which catalyze the degradation of acetate or propionate. Results presented in the last sections are consistent with this hypothesis. A recovery in the efficiency of waste conversion to methane may have resulted at about four months following initiation but data were not available to make the calculation. After about four months of operation, acetate began to accumulate in significant amounts. An interesting possibility which might explain the acetate accumulation involves changes in the concentration of hydrogen gas (not measured during these experiments).

The following scenario is consistent with, but cannot be concluded from, our present findings. One to two months after initiation of the digester H_2 must have increased to a higher concentration in the 3.3 day retention digester, as evidenced by the saturated status of methane production reactions. However, the increase in population density of H_2-using methanogenic bacteria, which had occurred by the fourth month of operation, apparently resulted in a reduction of H_2 concentration as the reduction of $NaH^{14}CO_3$ to $^{14}CH_4$ could be stimulated by H_2 addition by about the third month of operation. As described in the introduction, H_2 controls both the oxidation of reduced volatile fatty acids (McInerney et al., 1979) such as propionate, and their formation by fermenting bacteria (Wolin, 1974, 1975, 1976). As a consequence of H_2 accumulation, an increased production and a decreased rate of oxidation of reduced volatile fatty acids (e.g., propionate) would be expected. As a consequence of a subsequent decrease in H_2, increased production of acetate, either from oxidation of reduced acids, or from increased production during fermentation, apparently occurred. The resultant accumulation of acetate points to an imbalance between production and consumption of acetate. Apparently acetate-using methanogenic bacteria were unable to increase in population density to acclimate to the increase in acetate production. During the preparation of this report, the digester failed as evidenced by sudden decreases in pH from 7.0 to 5.5, gas production rate from 5 to 0.75 liters/day and % CH_4 from 46 to 14%. Several observations were made which could be of value in indicating impending digester failure. In particular, long chain volatile fatty acids such as hexanoic acid accumulated, the efficiency of waste conversion to gases, gas production rate and % CH_4 markedly decreased, and pH decreased from 7.5 to 7.0 (see Table 8). Digesters operated at 10 day and 5 day retention times showed stability over nearly one year of operation as evidenced by similar waste conversion efficiencies at different times after initiation (Tables 8 and 14).

Two important points were learned in the long term studies of this stressed digester. A practical lesson was that based upon present technology, operation of a digester of 8.8% solids at a 3.3 day retention is not recommended. The second lesson is the suggestion that limitations to the increase in population density of methanogenic bacteria which consume acetate may prevent the long term acclimation of a digester, operated under loading stress, to a stable efficient waste conversion process. Because of the benefits to operational and capital expenses of operation at short retention time, the limitations to increase in population density should be investigated. If, for example, the limitation were based on the nutrient environment of the digester, identification of the limiting nutrient and its supplementation could result in the long term acclimation of short retention digesters.

II. Methanogenesis in Natural Thermophilic Algal-Bacterial Mats

A. Temperature Relations of Anaerobic Decomposition

1. Upper temperature limit for anaerobic decomposition and methanogensis in Octopus Spring.

It was previously reported (first year completion report) that methane production at all temperatures of the algal-bacterial mat was increased by incubation at elevated temperature (65-70 C). Inability to grow at elevated temperature was not the basis for restriction of optimal methanogenesis to temperatures below the upper limit for the algal-bacterial mat (about 70 C). This observation was confirmed in a similar experiment in Octopus Spring (Figure 2, appendix B). The inclusion of higher incubation temperatures permitted the examination of the upper temperature limit for processes involved in anaerobic decomposition to methane. Above 70 C methane production was lower in samples collected from various temperatures in the mat. Although some methane production is indicated for the 50 C samples incubated at 75 C methane did not increase with incubation time and no methane production (above background levels) was observed at 80 C. Thus the upper temperature limit for anaerobic processes involved in conversion of algal-bacterial cells to methane appears to be 70-75 C.

Attempts to acclimate mesophilic waste conversion systems to higher temperatures have only been successful at temperatures below 60°C (Brown, et al., 1976; Cooney and Wise, 1975; Varel, et al., 1977). The ability of the natural thermophilic microflora involved in anaerobic decomposition to methane to be optimally active at 65-70°C may be advantageous in high temperature conversion processes. However, other results presented below indicate that novel applications may be necessary to achieve a successful waste conversion with the hot spring microorganisms.

2. Isolation of methanogenic bacteria at various temperatures.

In order to study the temperature relations of methanogenic bacteria it was necessary to first isolate methanogenic bacteria from the algal-bacterial mat. As it was not important to select any particular type of methanogenic bacterium we decided to attempt enrichment and isolation of H_2-utilizing methanogenic bacteria. Methanobacterium thermoautotrophicum had previously been isolated from this type of enviroment (Zeikus, 1977). Details of the enrichment and isolation of methanogenic bacteria from 50, 55, 60, and 65°C regions of the Octopus Spring mat, at the temperature of sample collection, are provided in appendix B. All isolates were long, irregular, Gram positive, rod-shaped bacteria which exhibited a blue-green autofluorescence. Hydrogen, but not formate, acetate or methanol served as an energy source for growth. Growth and methane production were both optimal for all isolates at 65 C (Figure 4, appendix B). Methane production and growth occurred for all isolates, except the 50 C strain, at 70 C. No growth or methane production was observed at 75 or 80 C. Except for a vitamin B_{12} requirement all isolates appear similar to M. thermoautotrophicum (Balch, et al., 1979; Bryant, 1974; Zeikus and Wolfe, 1972). Methanogenic bacteria in this environment apparently have not adapted to grow optimally at the temperature to which they are constantly exposed. The existence of such temperature strains has been reported for a variety of thermophilic organisms and processes (see Brock, 1978) and in particular for organisms and processes in algal-bacterial mats of alkaline silicious hot springs (Bauld and Brock, 1973; Peary and Castenholz, 1964; Brock, 1967a; Brock and Brock, 1968).

3. Correlation of methanogenesis and primary productivity in Octopus Spring.

It was previously suggested that the restriction of primary productivity to lower temperatures would also restrict all processes dependent upon algal-bacterial organic matter, such as anaerobic degradation (Doemel and Brock, 1977) and methanogensis (Ward, 1978). Although a broad temperature optimum for primary productivity in algal-bacterial mats was reported (48.3-58.5 C, Brock, 1967b), differences in carbon dioxide specific activity were not accounted for. In our experiments (Figure 3, appendix B) we found that primary productivity was sharply limited at temperatures above about 50 C. There was reasonable correlation between primary productivity and methane production, both of which showed significant activity at only 50 and 55 C.

4. Temperature relations of sulfate reduction in a high sulfate algal-bacterial mat.

Ward and Olson (1980, appended to last completion report) showed that sulfate reduction dominated methane production as the terminal stage of decomposition in algal-bacterial mats of high sulfate hot springs. As a part of other research activities we were able to perform experiments in an Icelandic hot spring which confirmed the temperature relations of microorganisms involved in anaerobic decomposition. As in the case of methane production in low sulfate algal-bacterial mats, optimal sulfate reduction occurred at a temperature (50°C) well below the upper limit for mat growth (Figure 14). If, however, mat samples collected at 50°C were transferred to higher temperatures for incubation, sulfate reduction was increased. Optimal sulfate reduction occurred at 60-65°C. The temperature relations of bacteria involved in thermophilic decomposition in natural high temperature environments appear to be similar despite the difference in the terminal anaerobic process.

B. Carbon and Electron Flow Through Terminal Steps in Anaerobic Degradation

1. Fate of 2-^{14}C-acetate

When 2-^{14}C-acetate was used to study reaction 3 (Table 1) in methanogenesis it was difficult to demonstrate the formation of $^{14}CH_4$ (Table 1, appendix C). Even when 2-^{14}C-acetate was provided after methanogenesis was ongoing (48 hours after sampling) or at low levels of ^{3}H-acetate little or no radioactive methane was detected (Tables 2, 3, appendix C), suggesting the lack of importance of acetate in methane production in this environment. When ^{14}C was recovered in gaseous, cell and liquid fractions, it was observed that 2-^{14}C-acetate was rapidly taken up by cells (Tables 1 and 2, appendix C). The same phenomenon was observed in the algal-bacterial mats of high sulfate springs (Table 9). Even though we had previously reported the conversion of 2-^{14}C-acetate to $^{14}CO_2$ in Bath Lake (Ward and Olson, 1980) more acetate was incorporated than was converted to CO_2. The incorporation of acetate into cellular carbon was decreased by dark incubation suggesting that phototrophic microorganisms may be involved. Autoradiograms of labelled material were prepared so that incorporated ^{14}C could be associated with the type of cell involved. Results (Figure 3, appendix C; Figure 15) clearly indicated that long filamentous bacteria, and Mastigocladus laminosus, the dominant phototroph of the Iceland mat (Castenholz, 1969), incorporated most of the ^{14}C in cells. It is reasonable to guess that acetate was accumulated by the filamentous phototrophic bacterium Chloroflexus aurantiacus as 1) this organism is common to all of the algal-bacterial mats sampled, 2) results indicated the involvement of a phototrophic organism, and 3) Chloroflexus aurantiacus is able to grow photoheterotrophically using acetate as a carbon source (Sirevag and Castenholz, 1979; Pierson and Castenholz, 1974; Madigan, et al., 1974). Thus, in the algal-bacterial mat environment acetate does not appear to be a significant methane precursor. The proximity of active photoheterotrophic organisms and anaerobic processes (indicated by methanogenesis which was optimal at a depth of 2-4 mm in Octopus Spring (Ward, 1978); Doemel and Brock (1977) suggested that photosynthetic activity was possible only in the upper 1-3 mm), apparently leads to acetate consumption by photoheterotrophs rather than by methanogenic bacteria. A similar situation exists in the rumen and gastrointestinal fermentations where methane is produced mainly from carbon dioxide as the host animal absorbs volatile fatty acids for its energy metabolism (Mah, et al., 1977).

Another interesting and consistent observation was the reported absence of a thermophilic Methanosarcina (capable of acetate conversion to methane (Zinder and Mah, 1979)) in a similar hot spring algal-bacterial mat (Zinder, personal communication). We previously reported (second quarterly report for first year) that high numbers of acetate-utilizing methanogenic bacteria may exist in algal-bacterial mats. These studies were done using a medium which supplied a source of fermentable material which led to hydrogen production. Thus, methanogenesis could just as well have occurred from reaction 2 as reaction 3 (Table 1) in these enumerations. No blue-green autofluorescing sarcina have been observed to presumptively identify the thermophilic Methanosarcina in the algal-bacterial mats (see Mink and Dugan, 1977).

2. Relative importance of carbon dioxide as methane precursor in low sulfate mats.

The rapid conversion of $NaH^{14}CO_3$ to $^{14}CH_4$ was reported in the first year completion report (Figure 1, appendix C). When the specific activities of $^{14}CH_4$ and $^{14}CO_2$ were compared, the relative importance of carbon dioxide in methanogenesis was found to be 71 ± 24% (n=5) for the Weigert Channel mat and 80 ± 10% (n=10) for the Octopus Spring mat. The relative importance was similar in the presence of added hydrogen or acetate (Table 4, appendix C). These observations are quite consistent with the reported insignificance of acetate in methanogensis (preceding section). Most of the methane formed came from carbon dioxide (presumably by reaction 2). The addition of hydrogen or acetate was shown to increase the contribution of carbon dioxide or acetate in methane production, respectively, in lake sediment where both reactions are active (Winfrey and Zeikus, 1979). The insignificant effect of acetate on the relative importance of carbon dioxide is further evidence that acetate does not contribute significantly to methane production in the algal-bacterial mats. The relative importance of carbon dioxide reduction in methanogenesis was similar at all mat temperatures (Table 5, appendix C).

If all methane was produced from carbon dioxide in this environment a specific activity ratio of 1.0 would be expected. The observed ratios (always less than 1.0) may be caused by inaccuracy in analytical methods. Methane precursors other than acetate (i.e., methyl amines, (Weimer and Zeikus, 1978; Hippe, et al., 1979; Zinder and Mah, 1979); and, methyl mercaptan (Zinder and Brock, 1978) are thought to be converted to methane directly and not via carbon dioxide as an intermediate. The possible contribution of these compounds to methane production in the algal-bacterial mats should be examined. Zinder, et al., (1977) showed that anaerobic degradation of algal-bacterial organic matter from Octopus Spring led to the generation of sulfur organic compounds including methyl mercaptan. An alternate explanation for the low specific activity ratio is the biological preference for ^{12}C over ^{14}C which is well known for methanogenic bacteria (see Mah, et al., 1977), and, in particular, for M. thermoautotrophicum (Fuchs, et al., 1979; Games, et al., 1978).

NONTECHNICAL SUMMARY

I. Animal Waste Conversion to Methane

The results to date permit a better understanding of the regulatory role of methane production reactions in waste conversion processes, but leave several important questions unanswered. Digesters stressed by increased manure loading provided stable environments of varying degrees of efficiency of waste conversion to methane. Figure 16 summarizes the status of the digesters, conclusions of experiments on these digesters, and indicates reactions of possible importance which must be addressed in future experiments.

Step-wise increase in loading of manure increased methane production rate to more than three times that reported for digesters operated at conventional loading rates. This was accomplished without pretreatment of the manure, merely by controlling feeding of manure to maximize conversion rates. Stable efficient conversion of waste manure to methane occurred when digesters were operated at a retention time of five days and 8.8% solids concentration. By processing manure at short retention time greater volumes of waste could be processed more rapidly, or the volume of a digester could be significantly reduced without decreasing the output of methane per unit time or per unit waste. This could significantly reduce the capital cost and affect time required to process a given amount of waste in a manure digester.

Further reduction in retention time (3.3 day) did not increase methanogenic rate and decreased the efficiency of waste conversion to methane. The deficit in methane production was shown to be balanced by volatile fatty acids which were removed daily from the unbalanced digester. Thus, the efficiency of waste conversion to products was constant upon increased loading, but the products formed were qualitatively different. In fact, at a 3.3 day retention time more energy from the waste manure fed was contained in the volatile fatty acid propionate than in methane. This could be of practical advantage if volatile acids are an important product (e.g., as chemical feedstocks or as animal feed).

The inefficient digester was a stable environment in which the basis for volatile acid accumulation (related to digester failure) could be studied. Methane production reactions were shown to be saturated (slashes over methane production reactions in Figure 16, bottom) and appeared to be the step which was rate-limiting for waste conversion to methane. The resultant increase in methanogenic substrates (acetate (and presumably hydrogen)) would be expected to have an inhibitory effect on propionate degradation as predicted in earlier work in our laboratory (first year completion report). However, propionate degradation occurred at similar rates in all digesters. The probable explanation for volatile fatty acid accumulation is the effect of methanogenic substrates (acetate and H_2) on fermenting bacteria, leading to increased propionate production. Preliminary methods for addressing this question in a subsequent grant period have been developed.

The long term acclimation of the methanogenic bacteria which become rate-limiting for rapid, stable, efficient waste conversion to methane, may represent a means by which natural limitations to digester operation may be overcome. Preliminary results suggest some acclimation by methanogenic bacteria to stressed operation, but further work is needed to explore this possibility.

II. Methanogenesis in Natural Thermophilic Algal-Bacterial Mats

Natural thermal environments contain bacteria which are adapted to degrade organic matter to methane at 65-70°C. This is 5-10°C higher than the maximum temperatures at which low temperature microbial populations have been adapted to convert waste to methane. The use of the natural thermophilic anaerobic microflora in conventional waste biodegradation systems would, however, probably be unsuccessful. As described above, the inefficiency and/or failure of such systems depends on a balance between the production and consumption of fermentation intermediates. In the algal-bacterial mat environment bacteria which usually carry out the conversion of acetic acid to methane appear to be inactive and probably absent. Their function in acetate removal is carried out by photosynthetic microorganisms which recycle acetate (summarized in Figure 17). In a dark digester acetate would probably accumulate causing failure due to pH decrease.

An interesting possible application might be a thermophilic digester operated at low light intensity so that the photosynthetic recycling of acetate could occur to prevent pH decrease. Such a digester would have the advantage that light energy would be converted to methane at high temperature as new algal-bacterial organic matter is degraded.

CONCLUSIONS

1) Under normal operating conditions for waste conversion systems (e.g. 15-30 day retention, 8% volatile solids) methane production occurs at suboptimal rates because methane production reactions do not receive a sufficient supply of substrate.

2) Increasing volumetric loading by decreasing retention time stimulates waste conversion to methane when retention time is shortened to 5 days. Waste is converted to methane at about the same efficiency but at over three times the rate of a digester operated at a conventional loading rate. No pretreatment of waste is required. The result may have a significant positive effect on either 1) the rate of which waste can be processed in a digester, or 2) reducing the volume of a digester needed to process a given amount of waste.

3) Operation at a retention of 3.3 days provided a digester which was inefficient with respect to waste conversion to methane. The relative stability of this digester provided a convenient model for determining the microbiological basis for inefficient digester operation and 2) whether changes in the numbers of microorganisms with time could have potential in terms of improving the technology of efficient waste conversion to methane. The long term instability of operation at a 3.3 day retention suggests that this operating condition is beyond the limitation of present technology.

4) Studies of the inefficient digester lead to the following conclusions:
    a) Waste conversion to fermentation products occurred efficiently so that hydrolytic and fermentation steps were not rate-limiting.
    b) Methane production reactions became saturated for substrate and were thus rate-limiting for waste conversion to methane.

   c) Accumulated substrates for methane-producing bacteria are the probable cause for volatile fatty acid accumulation.
   d) A 3.3 day retention probably did not exceed the ability of critical microorganisms to grow and prevent washout from the digester.
   e) Population changes occurred which may have resulted in increased waste conversion efficiency.
   f) Results implicate acetate-using methanogenic bacteria as a group whose population size may become limited, preventing long term acclimation of the digester to a 3.3 day retention time.
5) Natural low sulfate thermal environments harbor microbial populations which are adapted for conversion of organic matter to methane at 65-70°C. This could extend the upper temperature limit for thermophilic waste conversion processes by up to 10°C.
6) Methane-producing bacteria which catalyze removal of acetate (critical in maintaining balanced operation) do not appear to be active in the natural thermal environment due to the accumulation of acetate by photosynthetic microorganisms present in the same environment. Thus, novel approaches to the application of the natural thermophilic microflora to biomass conversions to methane, would be required to prevent digester failure.

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Table 1. Key reactions in the regulation of carbon and electron flow in waste conversion to methane (* indicates specific carbon whose fate indicates reaction mechanism) (see Thauer, et al., 1977 for equations and free energy changes).

Reaction 1 -- Propionate Degradation

$$CH_3CH_2C{*}OO^- + 3H_2O \longrightarrow CH_3COO^- + H{*}CO_3^- + H^+ + 3H_2$$

$\Delta G^{o'}$ = +18.2 kcal/reaction

Reaction 2 -- Hydrogen Reduction of Carbon Dioxide to Methane

$$4H_2 + H{*}CO_3^- + H^+ \longrightarrow {*}CH_4 + 3H_2O$$

$\Delta G^{o'}$ = -32.4 kcal/reaction

Reaction 3 -- Acetate Conversion to Methane

$${*}CH_3COO^- + H_2O \longrightarrow {*}CH_4 + HCO_3^-$$

$\Delta G^{o'}$ = -7.4 kcal/reaction

Table 2. Operating parameters for dairy cow manure digesters of varying retentiontime.

Retention Time	pH	gas production rate ml/l/hour	$\%CH_4$	μmoles CH_4/ml/h[a]	volatile fatty acids (mM)[b]						
					acetate	propionate	isobutyrate	butyrate	isovalerate	valerate	total
10 day	7.5	80.4	57.3	2.06	5.87	0	0	0	1.84	1.08	8.79
5 day	7.5	131.9	55.1	3.24	9.1	41.44	0.62	0	1.0	0	52.16
3.3 day	7.5	138.5	52.6	3.25	15.77	69.44	5.28	0.42	6.23	1.71	98.86

[a] gas production rate x % CH_4 ÷ 22,400 ml/mole ÷ 1000 = μliters CH_4/ml/h.

[b] in digester sample removed before daily feeding

Table 3. Contribution of acetate in dairy cow manure fed to the acetate pool level.

Retention Time	Expected Increase in Acetate Pool[a]
10 day	4.33 mM
5 day	8.66 mM
3.3 day	13.12 mM

[a] 43.3 mM acetate x proportion of total volume fed daily. Date of the first year completion report.

Table 4. Efficiency of waste conversion to gases or to gases plus volatile fatty acids (VFA) in dairy cow manure digesters operated at different retentiontimes.

				mmoles VFA removed per day[d]							% efficiency	
Retention time	g Volatile solids[a] fed per day	ml gas produced[b] per day	g C in gaseous[c] products per day	acetate	propionate	isobutyrate	butyrate	isovalerate	valerate	g C in VFA per day[e]	gases[f]	gases + VFA's[g]
10 day	15.1	3860	2.07	1.17	0	0	0	0.37	0.22	0.063	34.3%	35.3%
5 day	30.2	6330	3.39	3.64	16.58	0.25	0	0.4	0	0.70	28.1%	33.9%
3.3 day	45.4	6647	3.56	9.46	41.66	3.17	0.25	3.74	1.03	2.18	19.6%	31.6%

[a] average dry weight (gm/ml) x % volatile solids in 200 ml (10 day), 400 ml (5 day), 600 ml (3.3 day) manure fed daily

[b] determined by average of 3-5 consecutive readings (24 h) on a recently calibrated wet test meter converted to ml gas/day

[c] ml gas /day ÷ 22,400 ml/mole x 12 g C/mole of CO_2 or CH_4

[d] mM levels of Table 2 x 0.2 liter, 0.4 liter or 0.6 liter removed daily from 10, 5 or 3.3 day retention digesters, respectively

[e] [mmoles acetate x 2 C/mole + mmoles propionate x 3 C/mole + mmoles (iso+n)-butyrate x 4 C/mole + mmoles (iso + n)-valerate x 5 C/mole] x 12 x 10^{-3} g C/mmole

[f] after Varel, et al. (1977): $\% \text{ efficiency} = 100 \times \frac{\text{g C in gases recovered/day}}{0.4 \times \text{g C in volatile solids fed/day}}$

[g] $\% \text{ efficiency} = 100 \times \frac{\text{g C in gases recovered/day} + \text{g C in VFA recovered/day}}{0.4 \times \text{g C in volatile solids fed/day}}$

Table 5. Comparison between carbon and electrons theoretically diverted from methane and carbon dioxide to volatile fatty acids at various volumetric loading rates in dairy cow manure digesters.

digester Retention time	mmoles C in gas[a]	theoretical[b] mmoles C in gas	mmoles C diverted[c]	mmoles C in VFA's[d]	% C[e] recovery	mmoles e diverted[f]	mmoles e in VFA/s[g]	e recovery[h]
10 days	172	172	0	---	---	---	---	---
5 days	282	344	62	60	96%	273	277	101%
3.3 days	297	516	219	181	82.5%[i]	922	851	92%

[a] data of Table 4 ÷ 12 g/mole C

[b] 2 times (5 day) or 3 times (3.3 day) the mmoles (in gas for the 10 day digester; assumes a constant percentage conversion in digesters fed at twice or three times the feed rate of the 10 day digester

[c] difference between theoretical and observed mmoles C in gas

[d] data of Table 4 ÷ 12 g/mole C

[e] mmoles C in VFA's/mmoles C diverted

[f] mmoles C diverted x % CH_4 x 8e/CH_4

[g] data of Table 4(mmole C_2 x 8 + mmoles C_3 x 14 + mmoles C_4 x 20 + mmoles C_5 x 26)

[h] mmoles e in VFA's/mmoles e diverted

[i] 38 mmoles C and 74 mmoles e were not accounted for; this could be any combination of products with an average C/e ratio of 0.5; formate would qualify and would not have been detected by our methods.

Table 6. Relative importance of carbon dioxide reduction to methane in the presence and absence of added hydrogen in dairy cow manure digesters of 10 day and 3.3 day retention times

Retention Time	Additions	SA_{CH_4}/SA_{CO_2}[a] at hour				
		1.5	2	3	4	4.5
10 day	none		---	0.098	0.24	
	+1 ml H_2		0.16	0.47	0.44	
	+2 ml H_2		0.41	0.55	0.47	
	+3 ml H_2		0.42	0.52	0.58	
3.3 day	none	0.12		0.13		0.12

[a] specific activity of CH_4 ÷ specific activity of CO_2 = fraction of methane derived from CO_2 x 100 = % relative importance of CO_2 in methanogenesis.

Table 7. Calculation of hydrogen concentration required to achieve $\Delta G'=0$ or $\Delta G'=-10.5$ kcal/reaction for propionate degradation in dairy cow manure digesters operated at various retention times.

Retention Time	H_2 concentration required for[a]	
	$\Delta G'=0$[c]	$\Delta G'=-10.5$ kcal/reaction[d]
15 days[b]	40 μM	0.14 μM
10 days[e]	40 μM	0.14 μM
5 days	150 μM	0.51 μM
3.3 days	150 μM	0.504 μM

[a] calculated from $\Delta G'=\Delta G^{0'} + RT\ln \frac{[\text{acetate}][HCO_3^-][H_2]^3}{[\text{propionate}]}$

where: $\Delta G^{0'}=+18.2$ kcal/reaction (Thauer, et al., 1977)
$R=1.987\text{x}10^{-3}$ kcal/deg mole
$T=310^{\circ}K$

[b] data from Appendix A of first year completion report

[c] required for exergonic conditions

[d] required for ATP conservation based on ATP hydrolysis at physiological conditions (Thauer, et al., 1977)

[e] propionate not detected; estimated at 1/10th acetate pool after 15 day turnover digester

Table 8. Conditions in dairy cow manure digesters after long term operation at various retention times.

Retention time	Days since initiation	pH	Gas Production (ml/day)	%CH_4	Volatile Fatty Acid Concentration (mM)							Conversion Efficiency[a]	
					Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	Hexanoic	gases	gases + VFA's
10 day	395	7.6	3780	61	3.7	0.3	--	--	--	0.02	--	33.5	35.2
5 day	290	7.5	6549	61	10.5	11.1	0.32	0.12	0.32	0.23	--	29.5	35.3
3.3 day	230	6.9	3857	45	61.3	38.7	3.9	12.6	2.7	2.5	4.1	11.4	34.8

[a] see footnotes to Table 4

TABLE 9. RECOVERY OF ^{14}C IN VARIOUS FRACTIONS FOLLOWING SHORT TERM INCUBATIONS OF 2-^{14}C-ACETATE WITH ALGAL-BACTERIAL MAT SAMPLES FROM VARIOUS HIGH SULFATE HOT SPRINGS.

CONDITION	% OF RECOVERED ^{14}C IN:			
	CO_2	CH_4	CELLS[a]	FILTRATE
A. BATH LAKE[b]				
light	11.6	0	56.6	31.8
dark[c]	1.8	0	40.4	57.8
label added 50 h. after sampling	2.9	0	2.9	94.2
B. ICELAND GRENDALSA, 50 C[d]				
light	11.9	0	29.4	58.6
dark	8.2	0	8.3	83.6

[a] Cells and filtrate separated by means of a 0.45 um Millipore filter.

[b] Average of duplicates; 7.5 h. incubation, except 5 h. incubation when label was added 50 h. after sampling.

[c] Dark conditions created by wrapping vials containing samples in aluminum foil.

[d] Average of duplicates; 2.5 h. incubation.

FIGURE 1. DETAILED VIEW OF REACTIONS INVOLVED IN ANIMAL WASTE CONVERSION TO METHANE

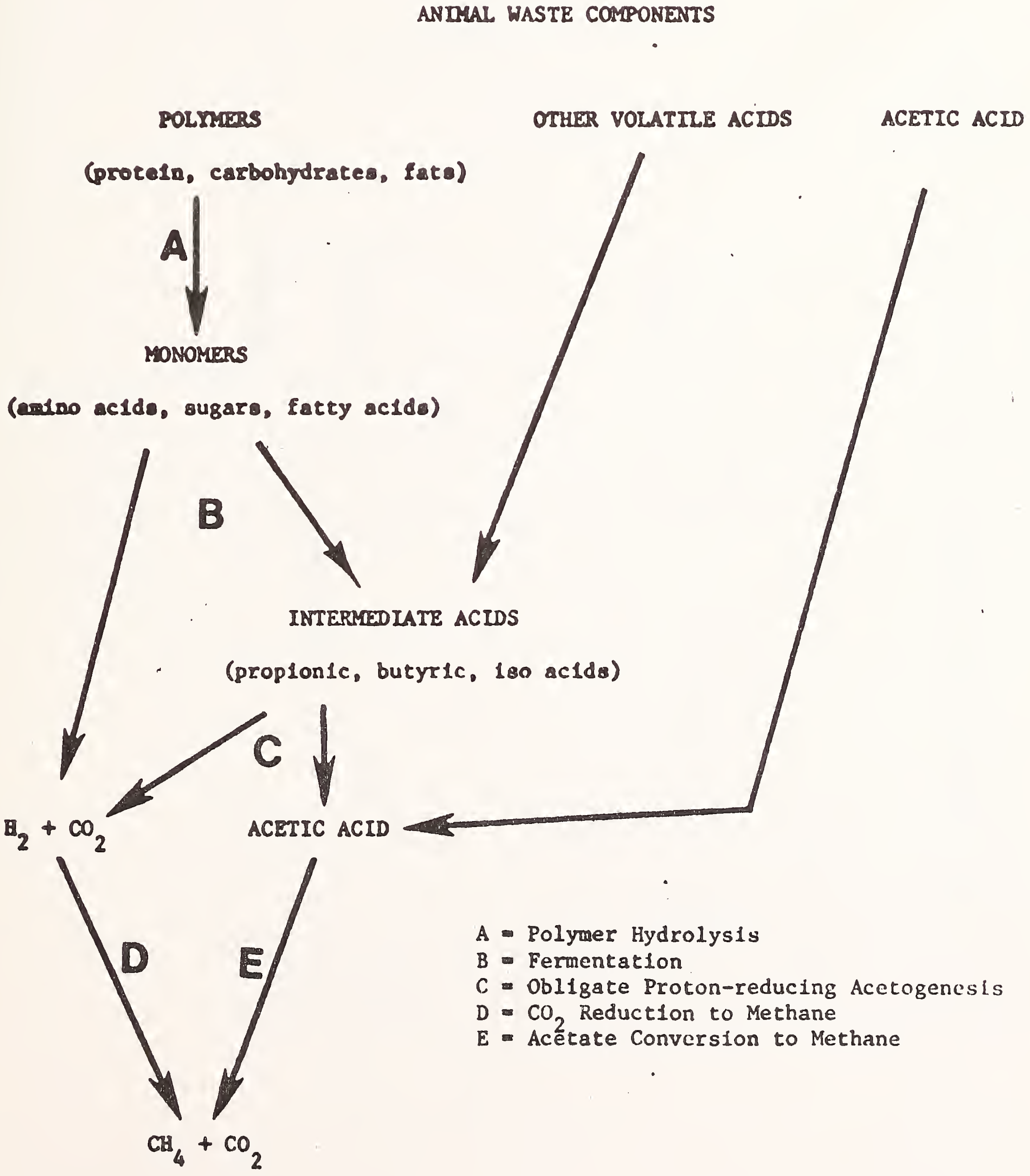

ANIMAL WASTE COMPONENTS
POLYMERS
(protein, carbohydrates, fats)
OTHER VOLATILE ACIDS
ACETIC ACID
A
MONOMERS
(amino acids, sugars, fatty acids)
B
INTERMEDIATE ACIDS
(propionic, butyric, iso acids)
C
$H_2 + CO_2$
ACETIC ACID
D
E
$CH_4 + CO_2$
A = Polymer Hydrolysis
B = Fermentation
C = Obligate Proton-reducing Acetogenesis
D = CO_2 Reduction to Methane
E = Acetate Conversion to Methane

Figure 2. Models for carbon and electron flow in dairy cow manure conversion to methane under A) balanced and B) unbalanced conditions. A slash through an arrow indicates saturation. Dashed lines indicate potential inhibition or saturation of reactions.

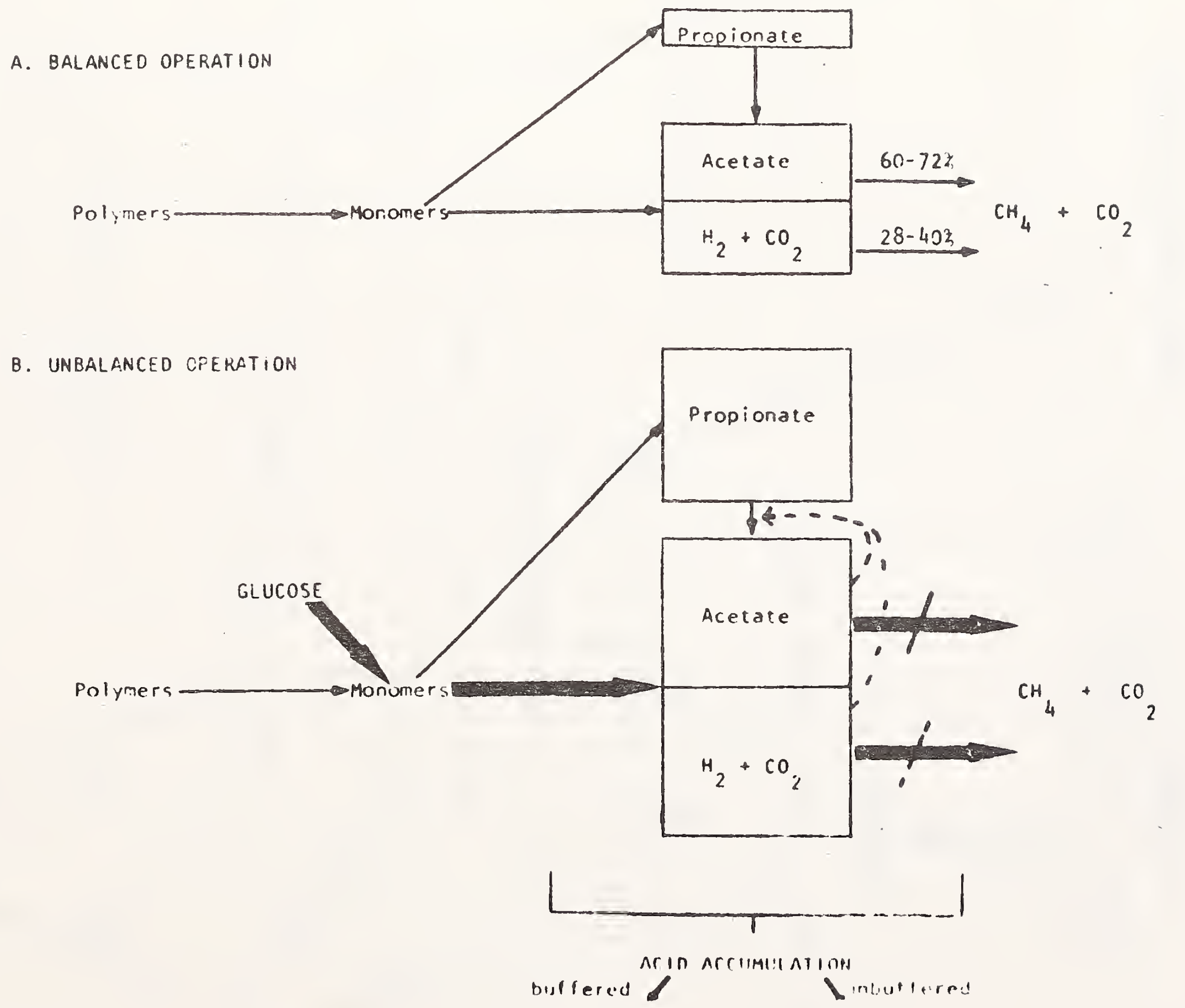


FIGURE 3. Effect of step down (indicated by arrows) in retentiontime from 10 days to 3.3 days in a dairy cow manure digester on A) gas production rate, and B) total volatile fatty acids.

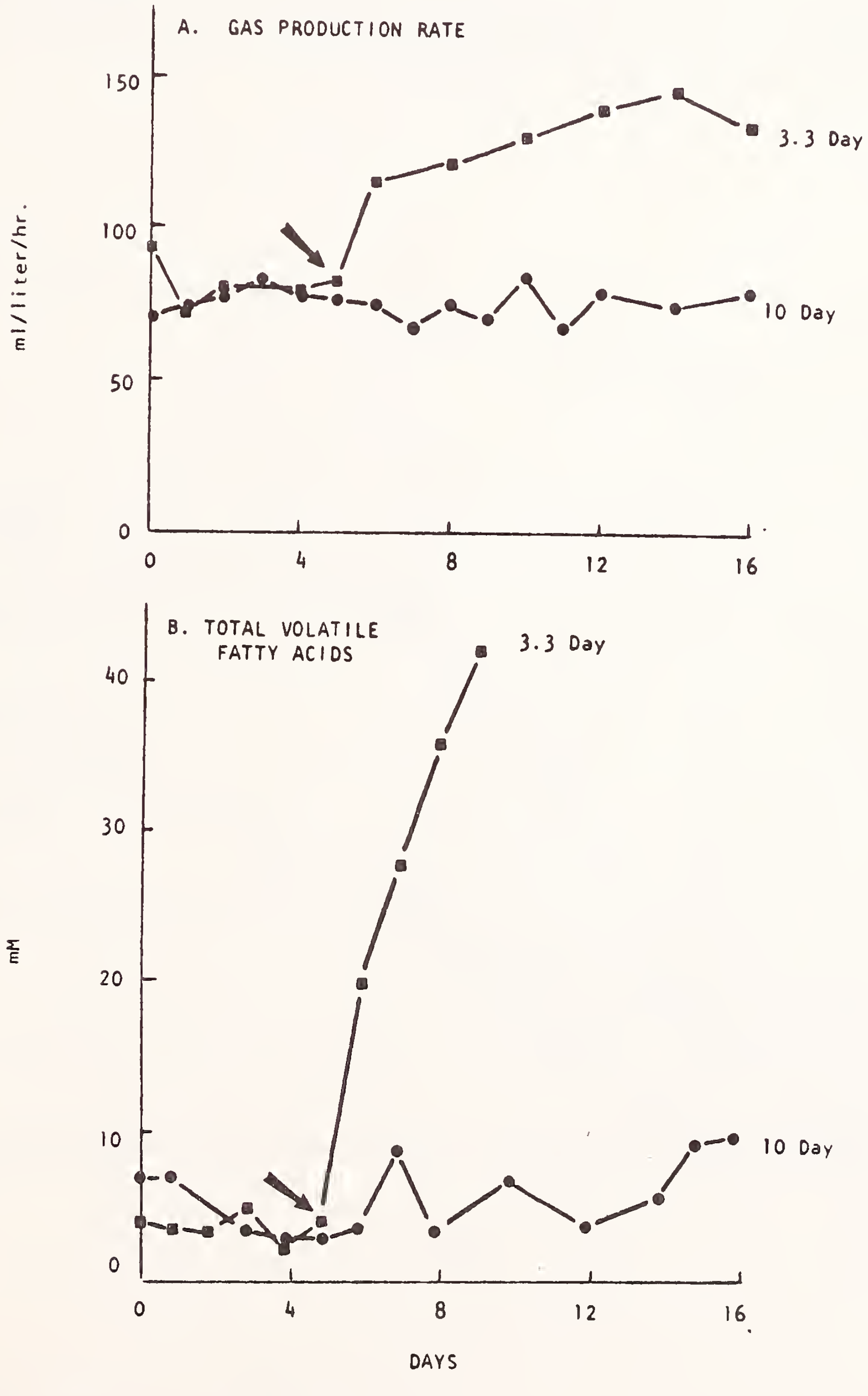


FIGURE 4. Volatile fatty acid levels in the contents removed daily from a dairy cow manure digester shifted from a retention time of 10 days to 3.3 days at day 0. Dashed lines indicate acetate increase immediately after feeding fresh manure, and projected decrease before the subsequent feeding.

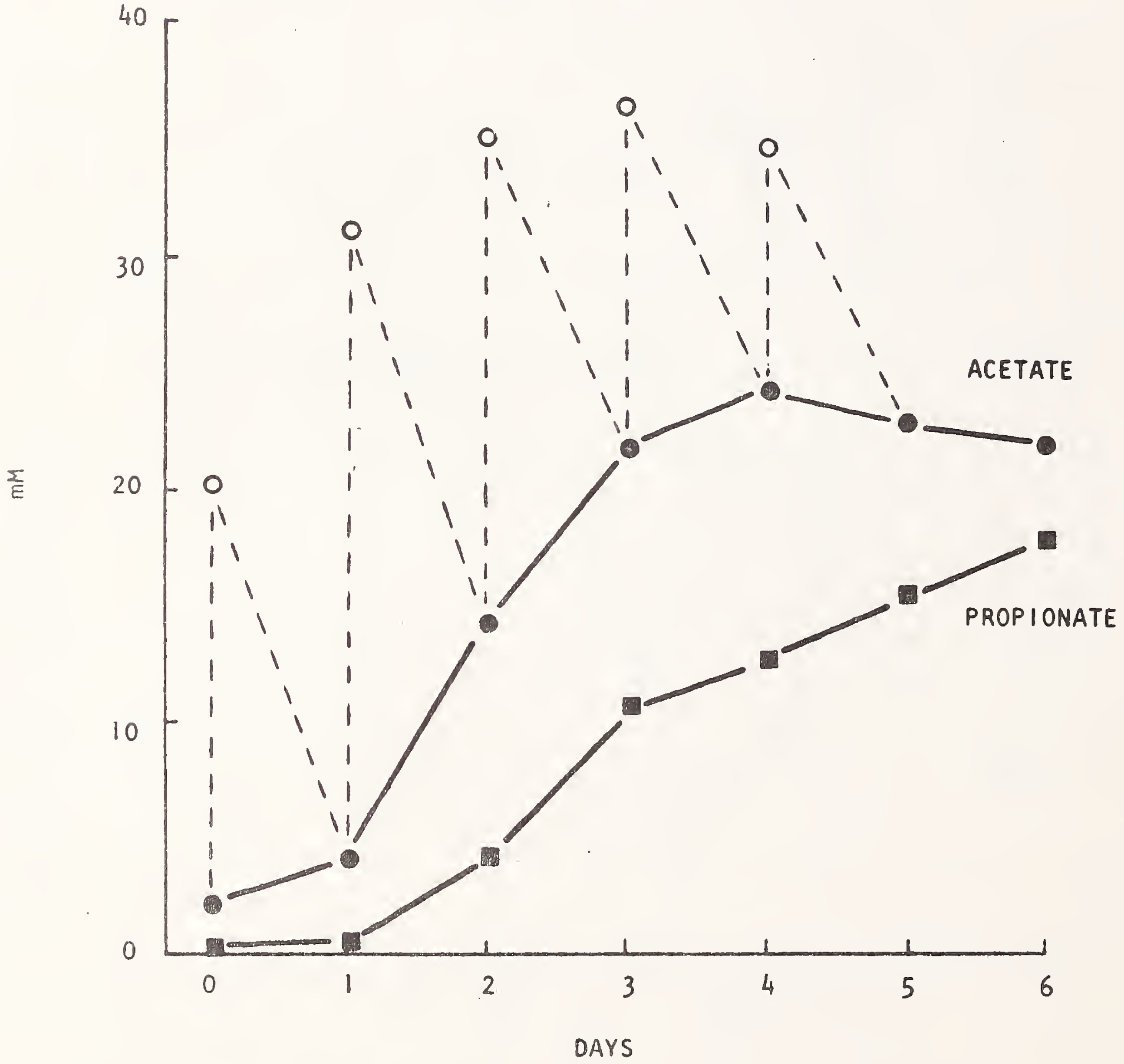


FIGURE 5. Changes in acetate (A) and propionate (B) after feeding dairy cow manure digesters operated at 10 day and 5 day retention times.

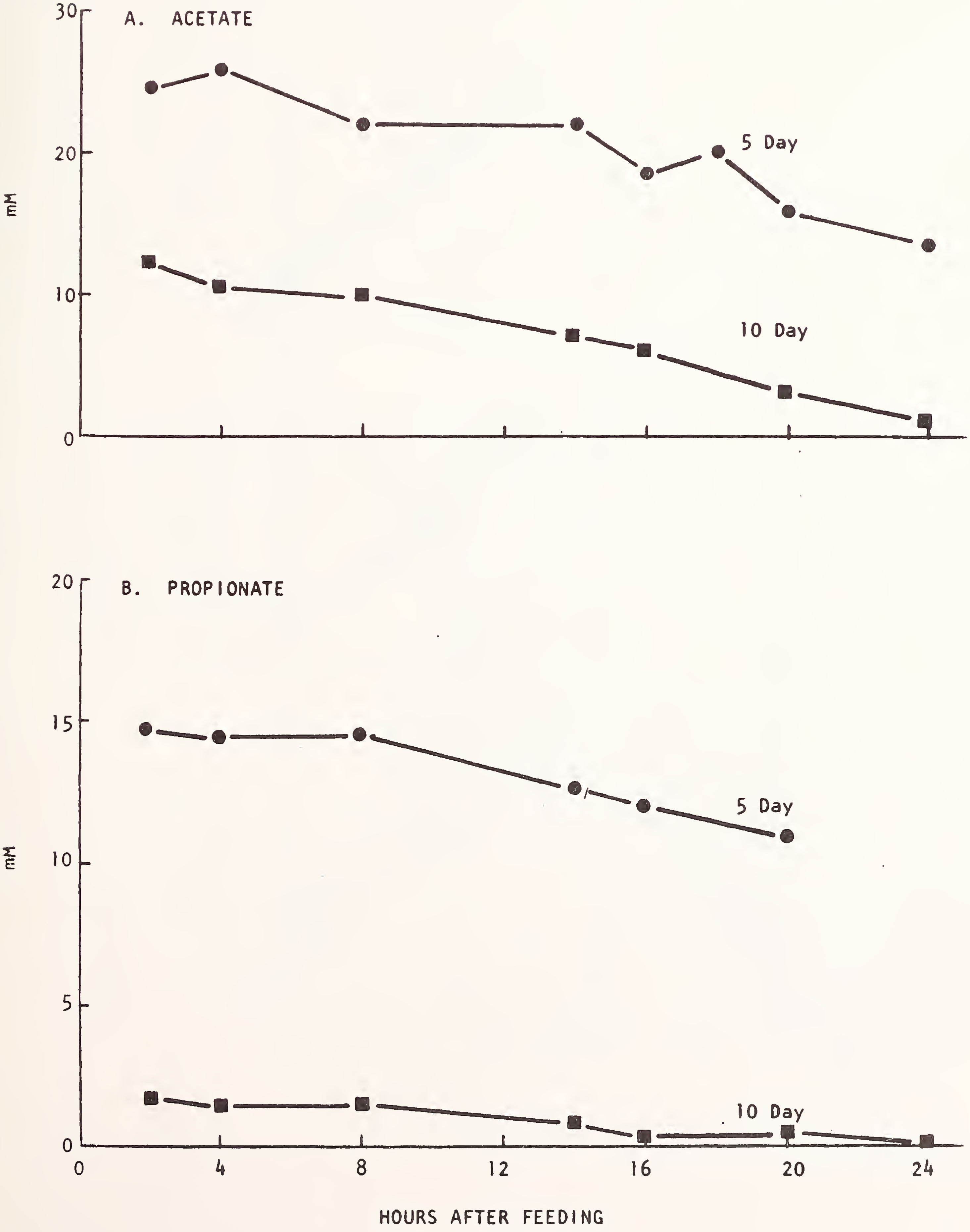
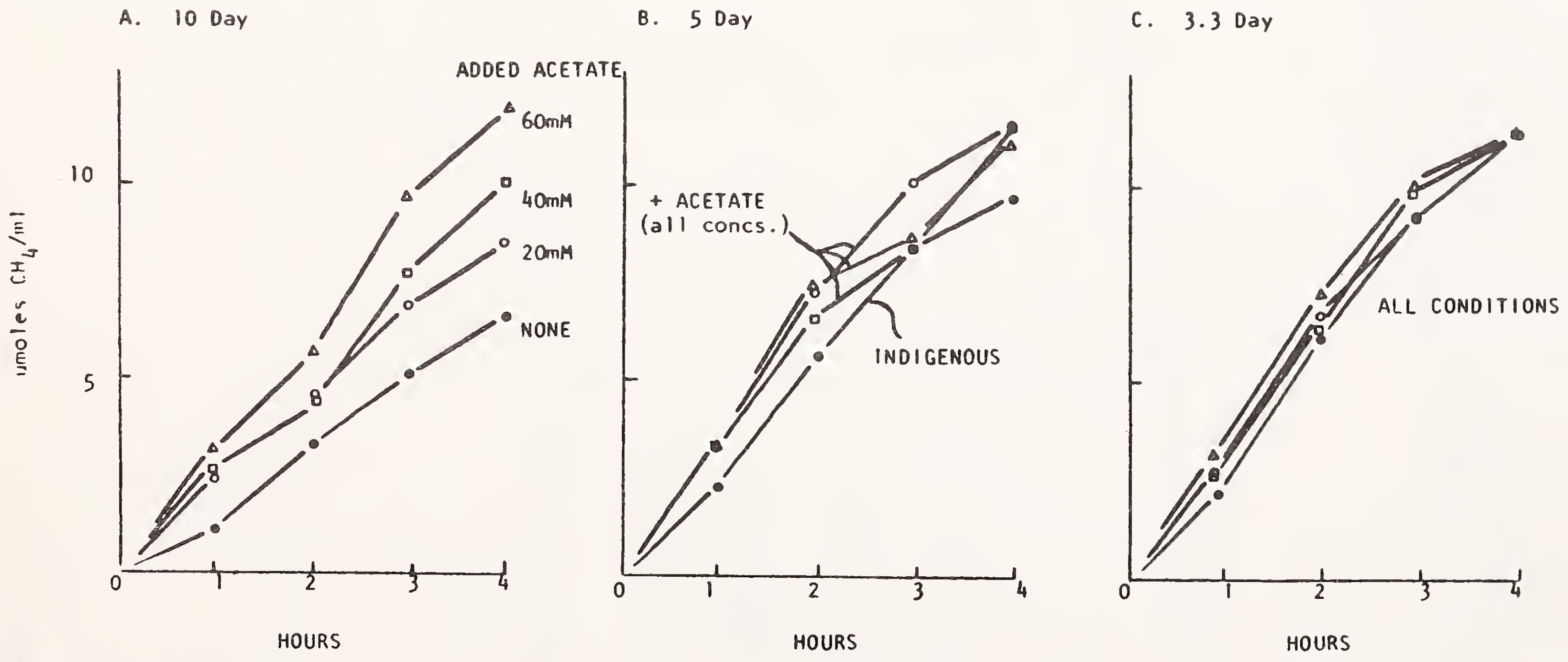


FIGURE 6. Effect of acetate on methane production in contents of dairy cow manure digesters operated at various retentiontimes.



INDIGENOUS RATE:

1.79 umoles CH_4/ml/h

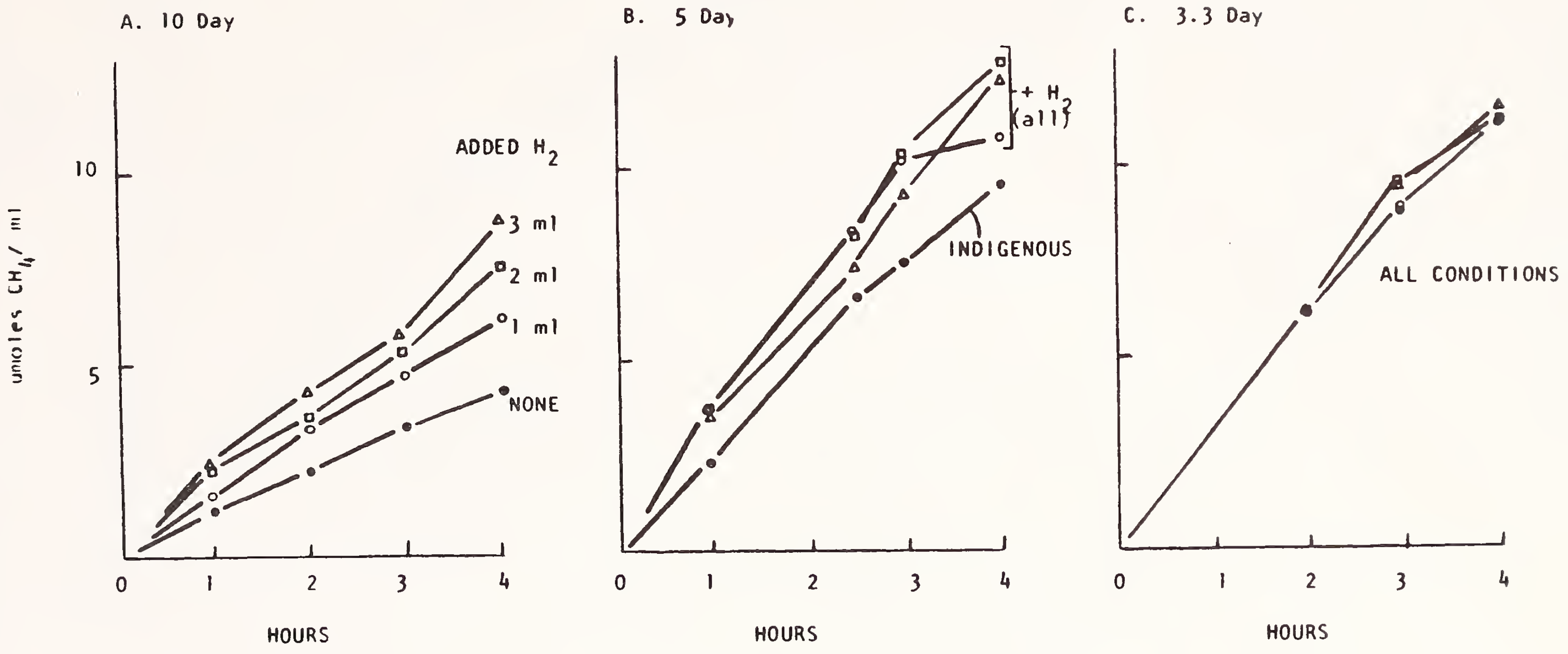
INDIGENOUS RATE:

2.6 umoles CH_4/ml/h

INDIGENOUS RATE:

3.12 umoles CH_4/ml/h

FIGURE 7. Effect of hydrogen on methane production in contents of dairy cow manure digesters operated at various retention times (H_2 added as 1, 2, or 3 ml 100% H_2 injected into a headspace of approximately 6 ml).



INDIGENOUS RATE:

2 06 umoles CH_4/ml/h

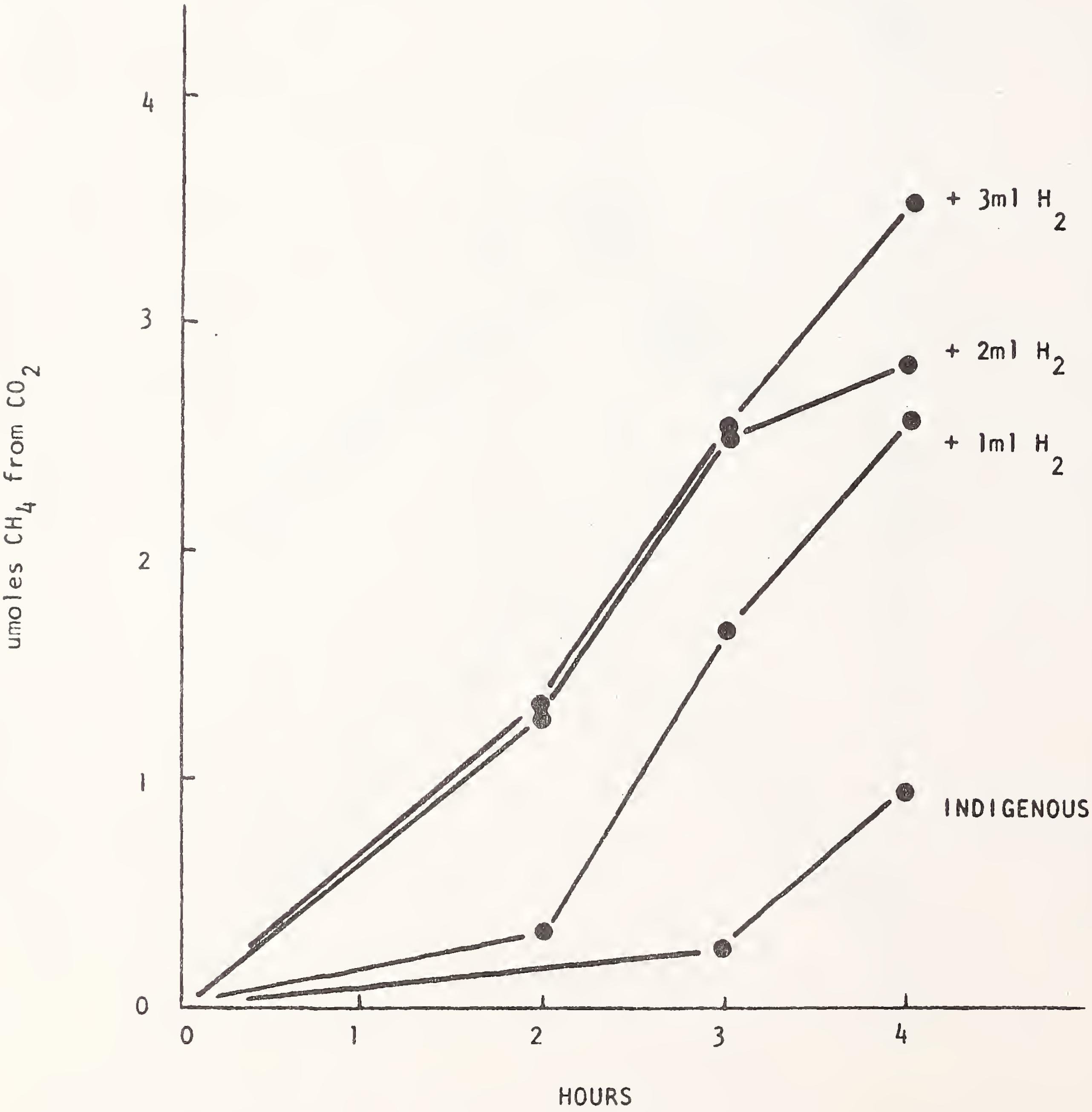
INDIGENOUS RATE:

2.4 umoles CH_4/ml/h

INDIGENOUS RATE:

2.62 umoles CH_4/ml/h

FIGURE 8. Effect of hydrogen on methane production from carbon dioxide (assayed by conversion of $NaH^{14}CO_3$ to $^{14}CH_4$) in a 10 day retention dairy cow manure digester.



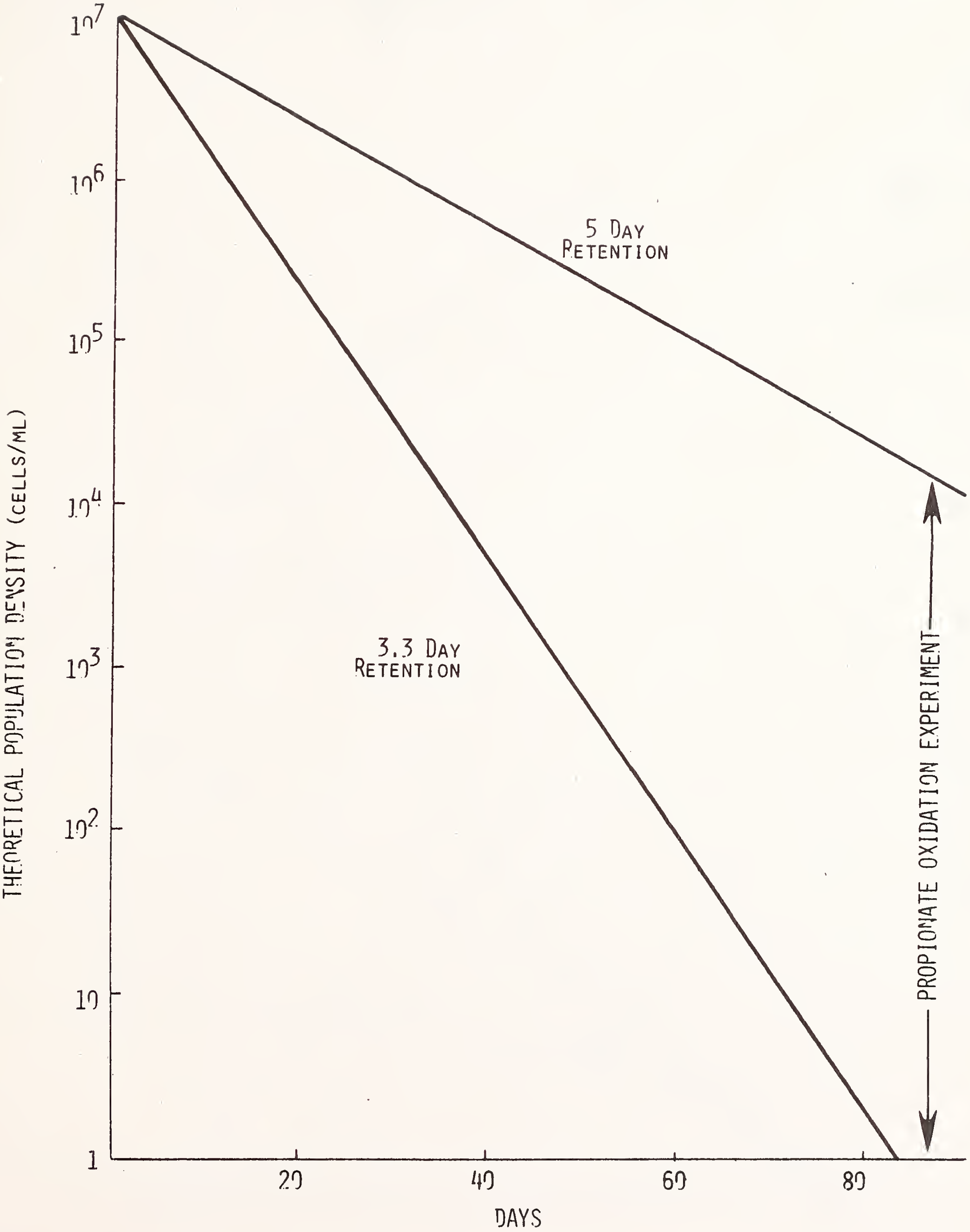
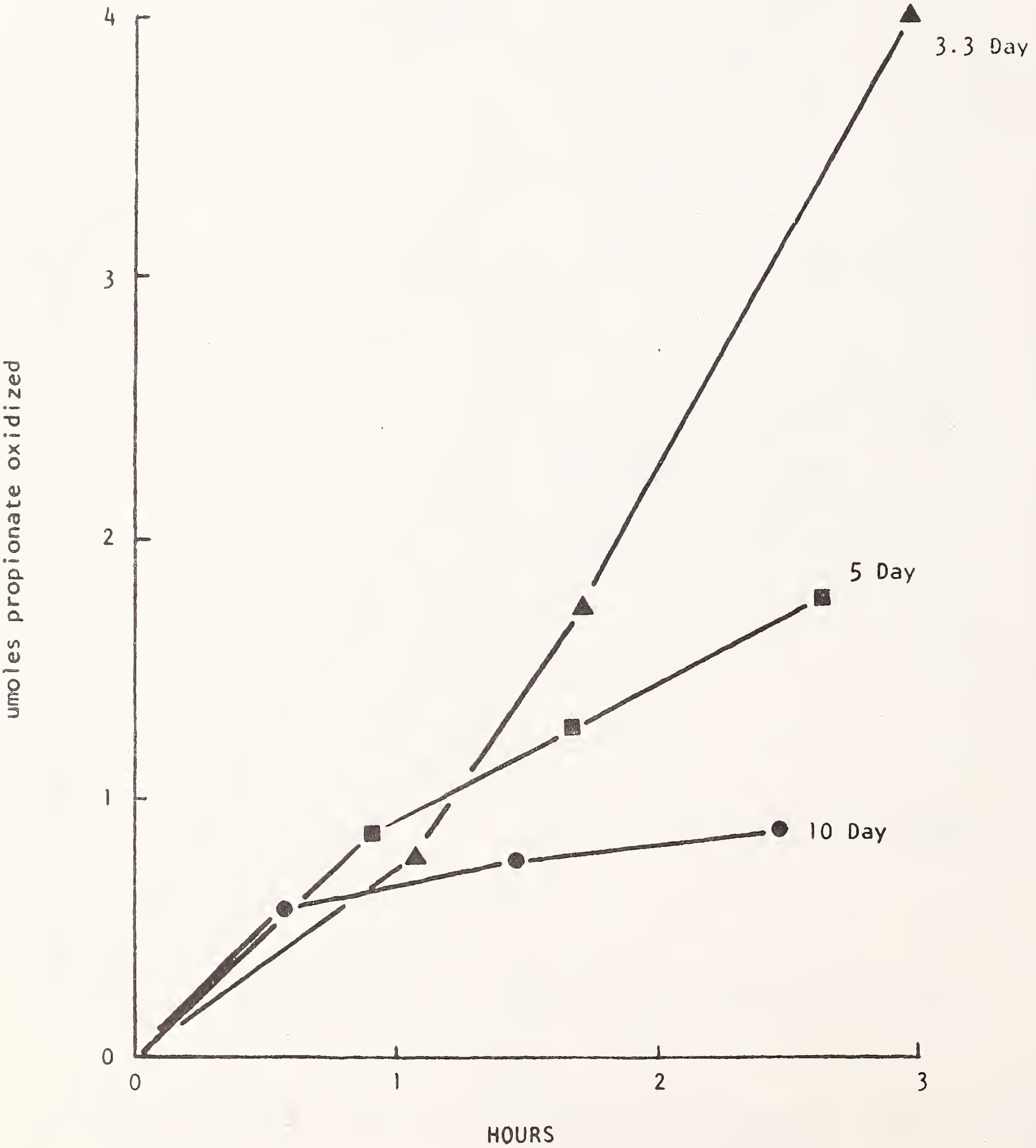


FIGURE 9. WASHOUT OF PROPIONATE-DEGRADING BACTERIA PREDICTED BY A MAXIMUM GROWTH RATE OF 0.19 DOUBLINGS/DAY (BOONE AND BRYANT, 1980) IN CONTINUOUS FLOW SYSTEMS OPERATED AT 3.3 AND 5 DAY RETENTION TIMES.

FIGURE 10. Propionate decarboxylation (assayed by conversion of 1-^{14}C-propionate to $^{14}CO_2$) in dairy cow manure digesters operated at various retention times.



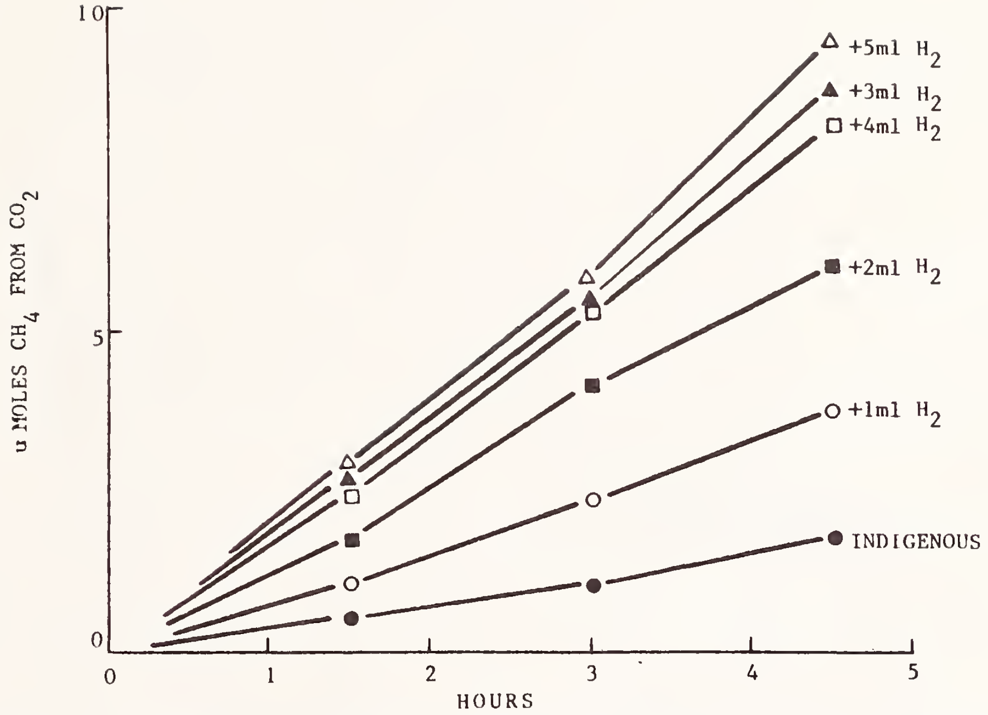


FIGURE 11. EFFECT OF H_2 ON $NaH^{14}CO_3$ REDUCTION TO $^{14}CH_4$ IN THE 3.3 DAY RETENTION DIGESTER AFTER 84 DAYS OF OPERATION.

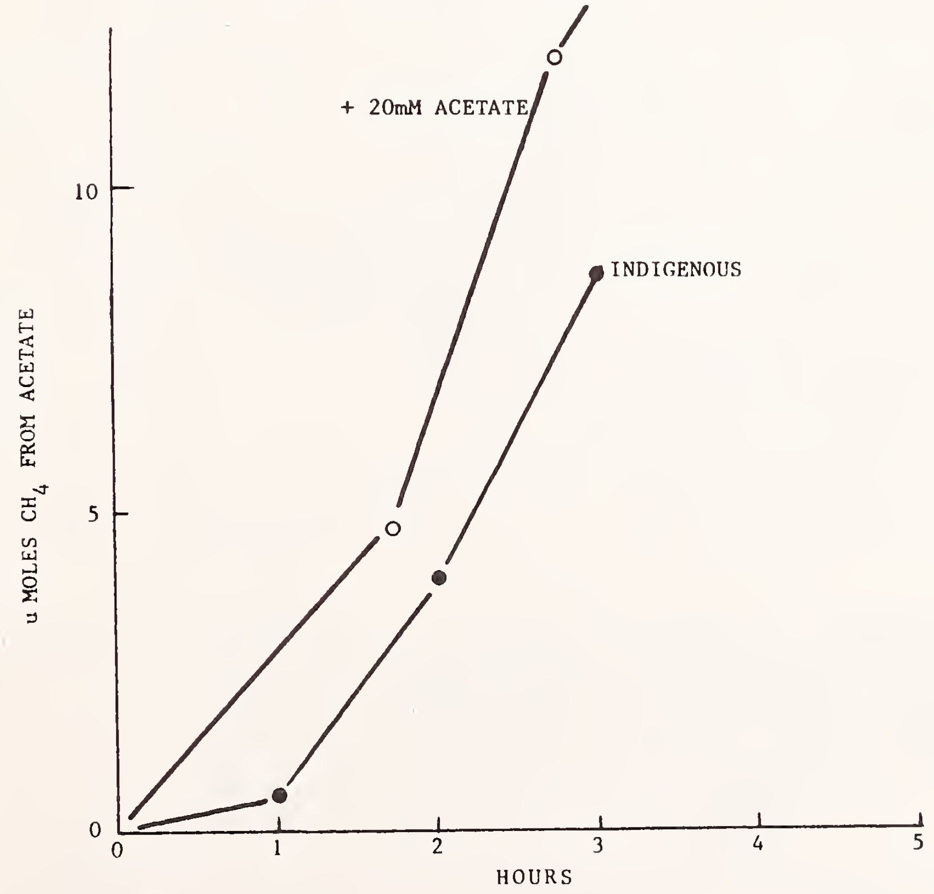


FIGURE 12. EFFECT OF ACETATE ON CONVERSION OF 2-^{14}C-ACETATE TO $^{14}CH_4$ IN THE 3.3 DAY RETENTION DIGESTER AFTER 134 DAYS OF OPERATION.

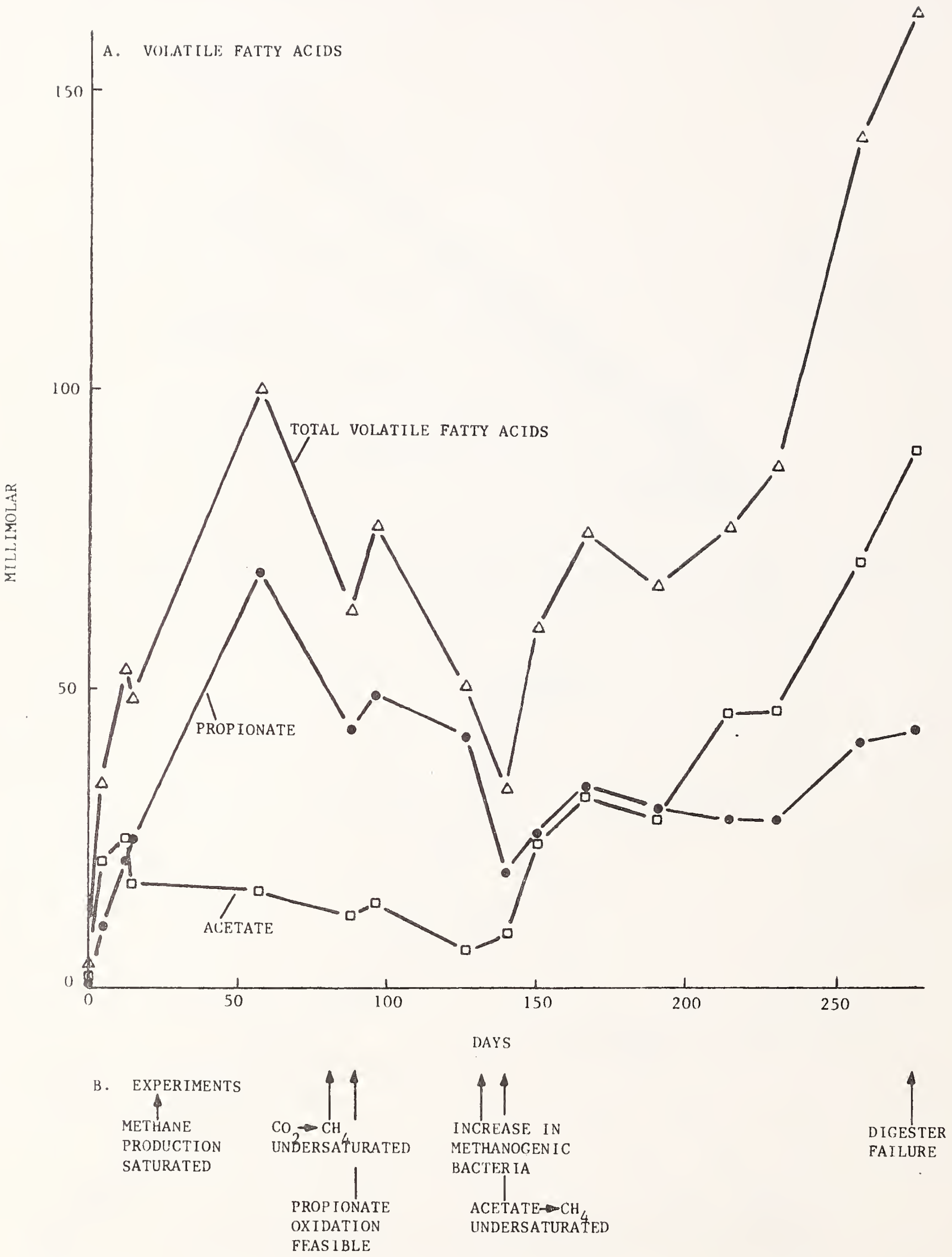


FIGURE 13. A. LONG TERM CHANGES IN VOLATILE FATTY ACIDS IN THE 3.3 DAY RETENTION DIGESTER.

B. SUMMARY OF MICROBIOLOGICAL FINDINGS IN THE 3.3 DAY RETENTION DIGESTER.

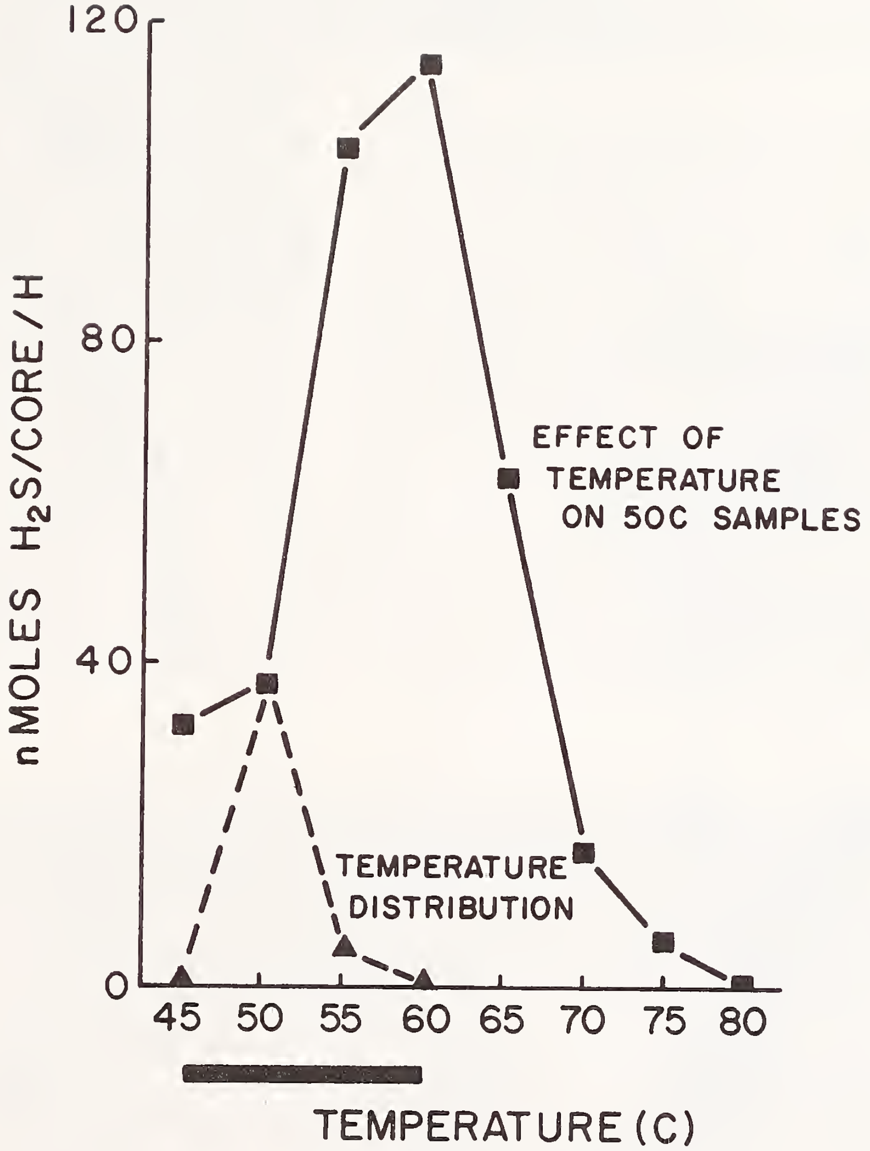


FIGURE 14. Temperature distribution of sulfate reduction in (dashed lines) and effect of temperature on sulfate reduction in 50 C samples (solid lines) collected from the algal-bacterial mat of the Iceland Grendalsa hot spring. Incubation was for 1.5 hours. Bar indicates the temperature range of the algal-bacterial mat.

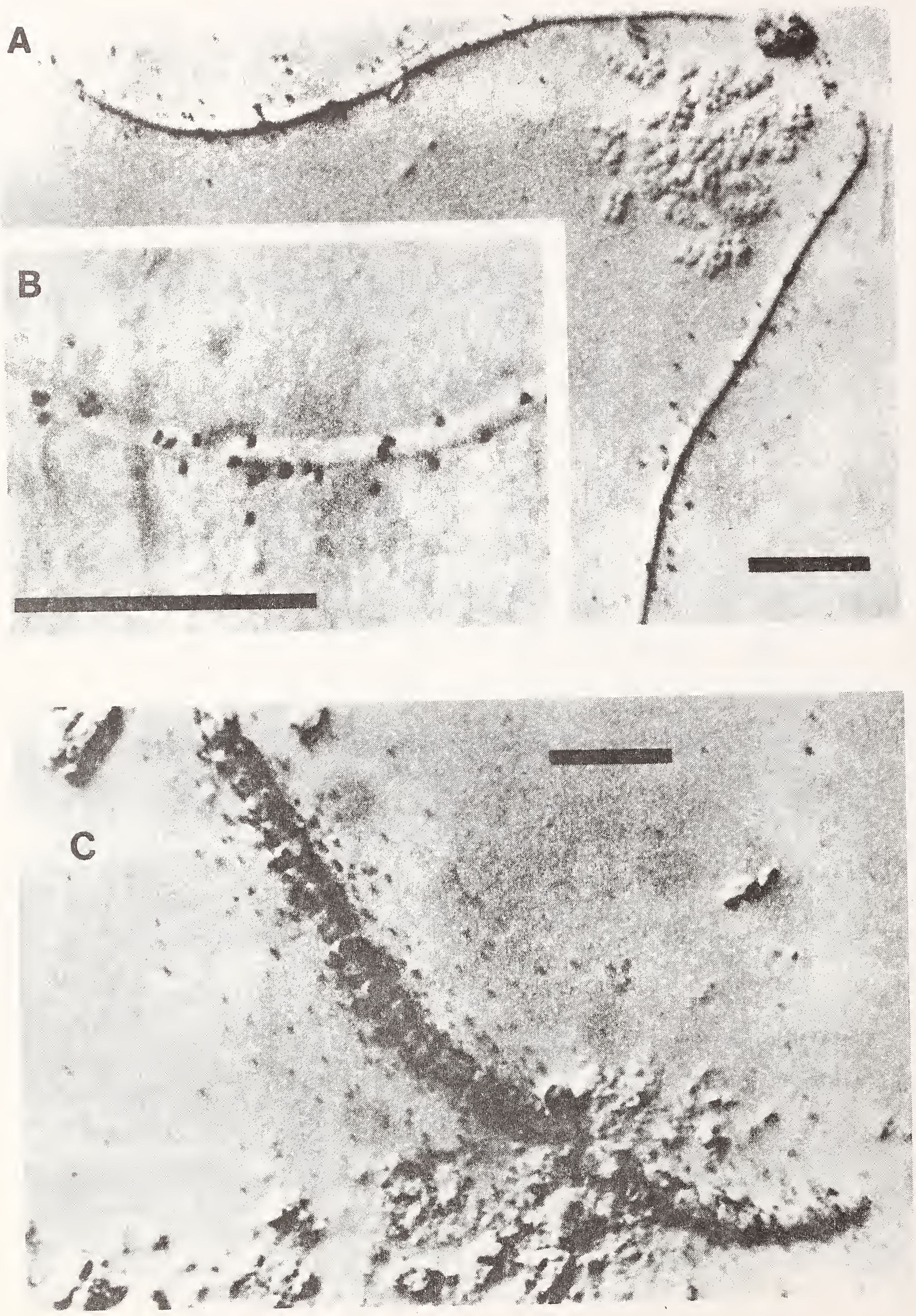
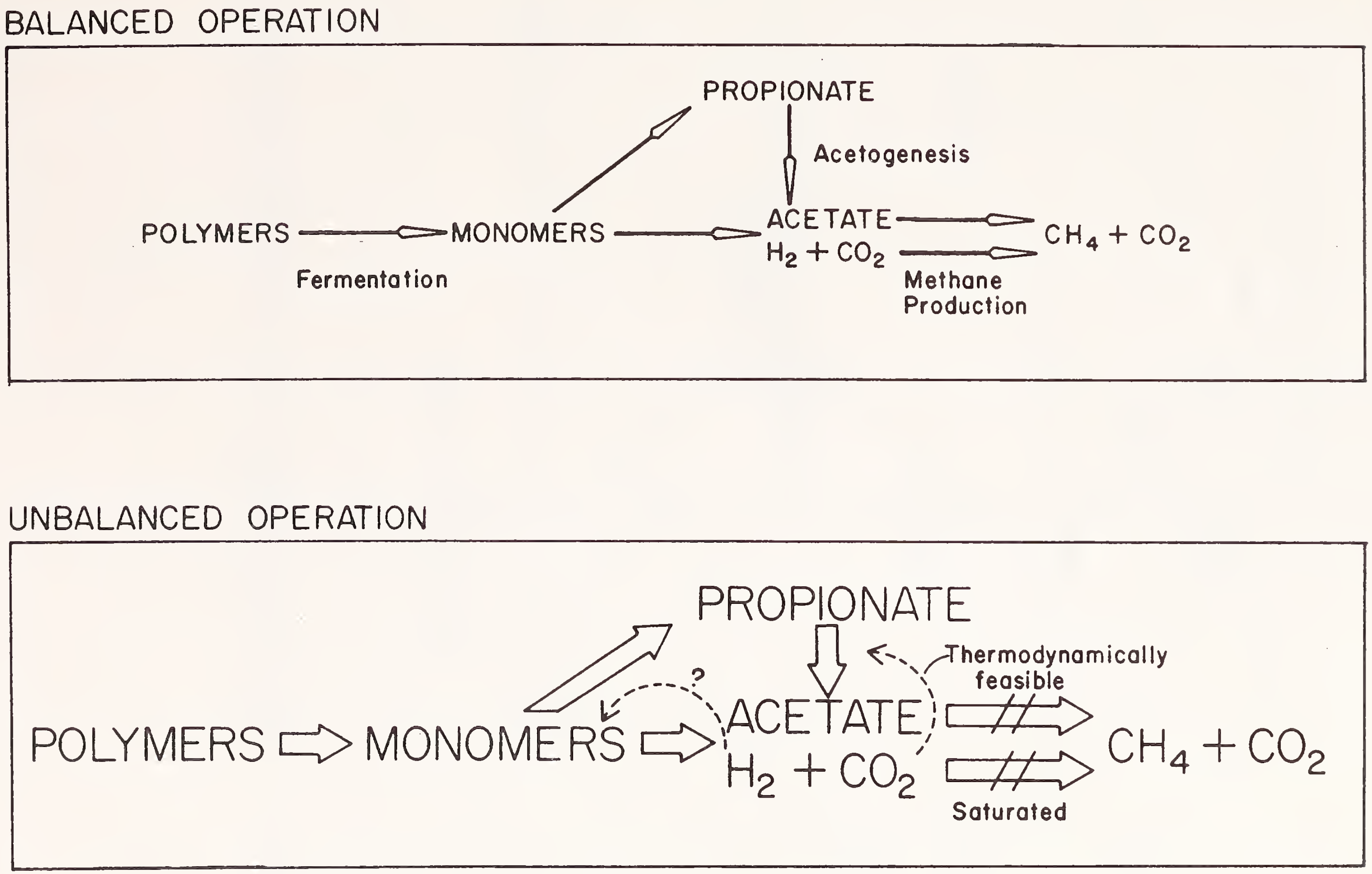


Figure 15. Autoradiograms of algal-bacterial mat samples from high sulfate hot springs after 2-7 hour incubation in the light with 2-^{14}C-acetate. (A and B) Bath Lake (C) Icelandic spring. Bars indicate 15 um.

FIGURE 16. Models for balanced and unbalanced operation of dairy cow manure conversion to methane.



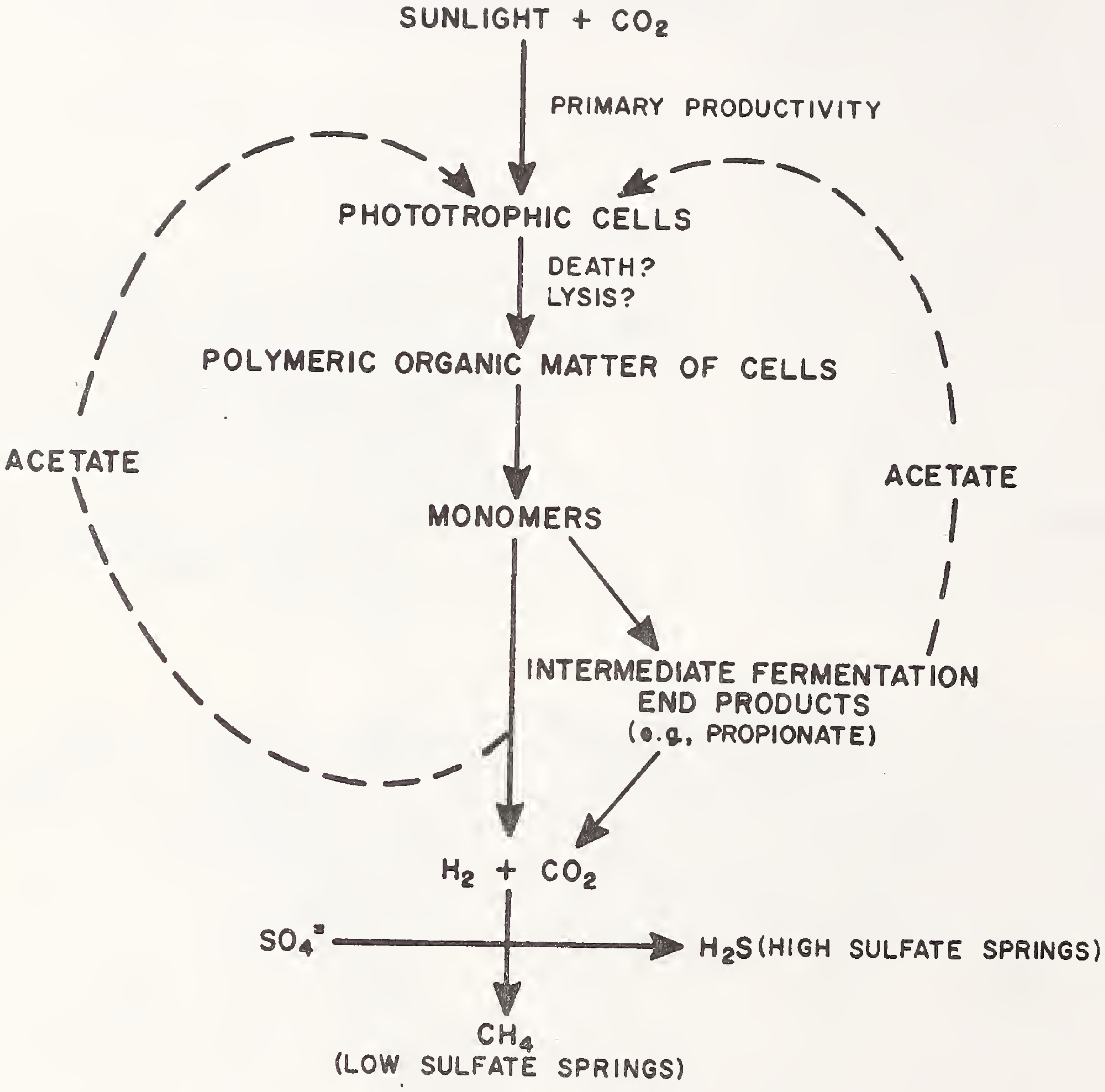


FIGURE 17. MODEL FOR THE PRODUCTION AND ANAEROBIC DECOMPOSITION OF HOT SPRING ALGAL-BACTERIAL MATS.

APPENDIX A. Accepted for presentation at the 2nd International Symposium on Microbial Ecology.

Conditions for Rate Limitation by Methanogenic Reactions in Dairy Cow Manure Digestion

David M. Ward, Eric Beck and William Rutherford

Dairy cow manure digesters were operated at different volumetric loading rates to achieve 10, 5 and 3.3 day turnover times of 8.8% dry solids (86% volatile solids) waste. Although methanogenic rate was increased by increased loading, the conversion efficiencies of waste to methane and carbon dioxide decreased from 34.3% to 28.1% to 19.6% in the 10, 5 and 3.3 day turnover digesters, respectively. When increased levels of volatile fatty acids (mainly propionate) were also included in calculations of fermentation efficiency, carbon and electron recovery was twice and three times as high in the digesters fed twice (5 day) or three times (3.3 day) the daily volumetric load of the 10 day turnover digester so that fermentation efficiencies were equivalent. Failure to stimulate methanogenesis by addition of hydrogen or acetate in the 3.3 day turnover digester confirmed that methanogenic reactions were saturated. Conversion of 1-^{14}C-propionate to $^{14}CO_2$, 2-^{14}C-acetate to $^{14}CH_4$, and the low relative importance of HCO_3^- in methane production (10-20%, measured by specific activity dilution) in all digesters, indicated that neither methanogenic bacteria nor propionate-decarboxylating acetogenic bacteria were washed out by decreased turnover time. Saturation of methane production reactions apparently led to a diversion of carbon and electrons to reduced volatile fatty acids, as hydrolysis and fermentation was proportionately increased by increased manure loading.

Appendix B. Submitted to Applied and Environmental Microbiology

TEMPERATURE RELATIONS OF METHANOGENESIS IN LOW SULFATE HOT SPRING ALGAL-BACTERIAL MATS

Kenneth A. Sandbeck and David M. Ward*

Department of Microbiology, Montana State University, Bozeman, Montana 59717

Running Head: Temperature and Methanogenesis in Algal Mats

ABSTRACT

Methanogenesis in algal-bacterial mats present in the effluent channels of low sulfate hot springs (Yellowstone National Park) was studied. Methanogenesis was found to be greatest 13°-23°C lower than the upper temperature limit for mat development which was about 73°C. Samples from various temperature regimes of the mat (50°, 55°, 60°C) all showed increased methane production upon incubation at elevated temperatures (65°-70°C). Methanogenesis was inhibited at 75°-80°C. Methanogenic bacteria isolated from various temperature regimes of the mat showed optimum methane production and growth at 65°C and an upper temperature limit at 75°C. The reason for maximal methanogenesis occurring below the upper temperature limit for mat development was not adaptation of methanogenic bacteria to lower temperatures. Methane production and primary productivity exhibited similar temperature distributions indicating methanogenesis might be limited by the availability of methanogenic precursors, the amount of which is probably a function of the rate of formation of algal-bacterial organic matter.

INTRODUCTION

Interest in the thermophilic conversion of organic wastes to methane has increased in recent years as thermophilic digestion of organic matter is more rapid and seems to be more efficient (8,15,16). Studies on decomposition in natural high temperature environments are potentially useful in understanding thermophilic bioconversion processes (22). The algal-bacterial mats present in the effluent channels of low sulfate hot springs provide such an environment. A thick (1-4 cm) mat develops between the upper temperature limit for photosynthesis (about 73°C) and the upper temperature limit for metazoan grazers of the mat (40°C) (3,7,19). Nearly complete decomposition of the algal-bacterial mat was suggested by Doemel and Brock (9). The isolation of Methanobacterium thermoautotrophicum from the Octopus Spring mat (21) and the observation of active methanogenesis in this mat (17) suggested that a complete anaerobic food chain was present. Previous reports indicated that maximum decomposition (9) and methanogenesis (17) occurred within or near the temperature range (48°-59°C) of maximal primary production (2,4). This study was undertaken to determine if inherent thermal limitations of the bacteria responsible for anaerobic degradation to methane restricted maximal methanogenesis to moderate temperatures (50°-60°C).

MATERIALS AND METHODS

Study Areas

The major research area used in this study was Octopus Spring, an alkaline hot spring (pH 8.0) located about 0.15 km SSE of Great Fountain Geyser in the White Creek drainage in Yellowstone National Park. At this spring, experiments were undertaken only in the southernmost effluent channel. Another research area used in this study was in a meadow, also in the Lower Geyser Basin of Yellowstone National Park, adjacent to Firehole Lake Drive. Springs in this meadow were collectively referred to as Serendipity Springs because of their chance discovery (10). The study area in the Serendipity Springs group was an artificial channel constructed by Fraleigh and Wiegert (10) by diverting the effluent of the spring (pH 6-7) so that it flowed at a constant rate down a plywood channel (1.2 m wide x 24 m long). Three other springs in Yellowstone National Park were also investigated initially to study methane production versus temperature. Two of the springs, Twin Butte Vista and Mushroom Spring were located about 0.10 km SE and 0.13 km NNE of Great Fountain Geyser, respectively. The third spring, referred to as West Thumb, is the first major spring located north of the West Thumb Geyser area that empties into Yellowstone Lake. Only the northernmost effluent channel of the West Thumb spring was sampled. All officially named springs in the Firehole Lake area are shown on maps in Brock (3).

Sampling

Whole cores were removed from the algal-bacterial mat with a no. 4 brass cork borer (50.3 mm^2) and transferred directly to one dram glass vials (Kimble 14.5 x 45 mm) which were sealed anaerobically (11, except that no copper reducing column was used in the field) under a stream of 100% helium (Linde). Recessed butyl rubber stoppers (A.H. Thomas, size 00) were used to effect a seal and keep the vials anaerobic during later manipulations. Stoppers were secured by lengthwise taping with masking tape. Anaerobically tubed samples were quickly placed in insulated coolers that contained water which was 5°C warmer than the *in situ* temperature. During transit to the laboratory (approximately 2 hours), samples cooled slightly, but this procedure ensured that samples remained within 5°C of their indigenous temperatures. In the laboratory, samples were transferred to dark incubators that matched the *in situ* temperature (except in the case of temperature transfer experiments where samples were incubated at several different temperatures). Gas samples were removed from the headspace periodically for methane analysis.

Primary Production

Replicate vials received 0.1 ml of a 20 µCi/ml stock solution of $NaH^{14}CO_3$ (44.5 mCi/mmol, New England Nuclear) diluted in sterile anoxic distilled water (pH 8.0). The samples were incubated in the effluent channel for 1.5 hours and biological activity was terminated by the addition of 0.5 ml formalin. The addition of formalin was accompanied by extremely vigorous shaking to ensure that the formalin permeated the gelatinous sample. "Light" replicate vials were taped only at the top so that upon incubation the core was exposed to sunlight.

"Dark" replicates were taped lengthwise and wrapped in aluminum foil to exclude light during incubation. At the laboratory, a gas headspace subsample was removed for determination of the specific activity of CO_2 (see below). After acidification with 0.1 ml 50% sulfuric acid to ensure removal of ^{14}C-carbonates, cores were dispersed using a teflon tissue homogenizer and radioactivity in 0.1 ml of the homogenate was determined in 10 ml Aquasol (New England Nuclear). A model LS 100-C liquid scintillation counter (Beckman) was used with the ^{3}H + ^{14}C window and a gain of 240 to determine cpm. Corrections for differences in counting efficiency were made by the automatic external standard method. The liquid volume of sample vials was determined by displacement with water so that results could be corrected to a per core basis. The specific activity of CO_2 (dpm/µmole) was divided into the dpm/core to convert results to µmoles of CO_2 fixed/core. Duplicate vials were then averaged and the amount of CO_2 fixed in darkened cores was subtracted from the amount of CO_2 fixed in the light to give light-stimulated CO_2 fixation. The cores differed in length, and since activity is not proportional to length (17), all results were reported on a per core basis.

Analytical Methods

Gas samples (0.2 ml) were removed from the headspace of vials using a helium flushed glasspak syringe (Becton-Dickinson) attached to a mininert valve (Supelco) (to minimize loss of sample due to pressure differences). CH_4, CO_2 and $^{14}CO_2$ were analyzed by gas chromatography-gas proportion analysis as described by Ward and Olson (18). Gas concentrations and radioactivity were quantified using a Spectra-Physics model 4100 computing integrator and a Spectra-Physics Minigrator, respectively. The total amounts of CH_4 were calculated by comparison of subvolume to gas headspace volume as determined by displacement with water.

Temperature was taken with a mercury thermometer.

Autofluorescence Microscopy

Blue-green autofluorescence was taken as presumptive evidence of methanogenic bacteria in methane positive cultures (13). Autofluorescence was observed in a Leitz Ortholux II microscope equipped with ultraviolet light vertical illumination from an HBO-200W mercury lamp that passed through a Leitz B cube excitation and emission filter combination.

Isolation of Thermophilic Methanogenic Bacteria

Enrichment, isolation and maintenance of thermophilic methanogenic bacteria was done in a basal medium (BM) that contained the following per liter distilled water (all chemicals used were reagent grade): KH_2PO_4, 0.15g; Na_2HPO_4, 1.05g;NH_4Cl, 0.53g; $MgCl_2 \cdot 6H_2O$, 0.20g; cysteine-HCl, 0.5g; .0001g resazurin and 10 ml of a trace mineral solution. The trace mineral solution contained per liter distilled water: nitrilotriacetic acid, 1.5g; $FeCl_2 \cdot 4H_2O$, 0.3g; $MnCl_2 \cdot 4H_2O$, 0.1g; $CoCl_2 \cdot 6H_2O$, 0.17g; $ZnCl_2$, 0.1g; $CuCl_2$, 0.02g; H_3BO_3, 0.1g; Na molybdate, 0.01g. The pH of the medium was adjusted to 9.2 so that the final pH was about 7.1 (±0.1) after all additions had been made. The medium was boiled under 100% helium (Linde), but dispensed under 20% CO_2/80% H_2 (Linde)(after 11) with a 5 ml Repipet (Lab Industries). Each roller culture tube (150 x 16 mm, Bellco) received 5 ml of medium and was fitted with a recessed butyl rubber stopper (A.H. Thomas, size 00). Roller culture tubes were autoclaved in a tube press. $Na_2S \cdot 9H_2O$ (pH 13) was added after autoclaving to achieve a final concentration of 0.03%. Since methanogenic bacteria have been shown to resist antibiotics that inhibit peptidoglycan synthesis (12), the isolation of the thermophilic methanogens was carried out with the sodium salt of either penicillin G (Eli Lilly), or ampicillin (Sigma) added to reach a final concentration of 300 μg/ml of medium. All additions to the medium after autoclaving were made from sterile anoxic stock solutions. BM was modified by adding 0.2% yeast extract and 0.2% trypticase for use in checking for heterotrophic contaminants (BM + TYE). A contaminant organism was isolated on BM + TYE (see Results) by dilution to extinction. A portion of a turbid culture of the contaminant that was grown in BM + TYE was sterilized by autoclaving, filtered through a 0.45 membrane filter (Millipore) and used to supplement BM (BM +S) so that methanogenic bacteria could be isolated by dilution to extinction. Growth in the highest dilutions containing methane and blue-green autofluorescing cells was serially diluted until 1) all cells exhibited similar morphology and fluorescence and 2) inoculation into BM + TYE yielded no growth. Isolates were obtained at 50°, 55°, 60° and 65°C using Octopus Spring algal-bacterial mat samples obtained to each of these temperatures as inoculum. To determine if growth could occur on formate, acetate, or methanol, these substrates were added to BM + S (1% final concentration) with helium in the gas phase.

Pure Culture Temperature Transfer Experiments

Experiments were performed with the isolates using BM + S to parallel temperature transfer experiments done with natural mat material. Each tube received a 0.1 ml inoculum from a turbid culture which had been pregrown in BM + S at the temperature at which it had been isolated. Each isolate was incubated at 50°, 55°, 60°, 65°, 70°, 75° and 80°C and methane production was followed in duplicate tubes over time. Replicate tubes were used to measure optical density (660 nm) after 70 hours relative to uninoculated medium on a Varian model 635 or a Gilford model 250 spectrophotometer (1 cm light path). Tubes analyzed for optical density were flushed daily with 20% CO_2/80% H_2 and given about 2.5 atmospheres of the CO_2/H_2 gas mixture via a 10 ml glasspak syringe fitted with a mininert valve. Sulfide was added after 48 hours (0.05 ml of a sterile anoxic 3% $Na_2S \cdot 9H_2O$ solution) because it was presumably lost through daily gas headspace flushings.

RESULTS

Methanogenesis Along the Thermal Gradient

Original experiments were designed to determine the region of greatest methanogenesis along the thermal gradients of several hot spring effluents. Although methane production was linear over the initial incubation period (48 hours), results were presented for the earliest time point rather than estimating a rate. Methane production in each of the five springs surveyed was greatest well below the upper temperature limit of the mat (Figure 1). The best temperature for methane production was 50° to 60°C.

Effect of Temperature on Anaerobic Degradation to Methane

When samples collected at various temperatures along the Octopus Spring thermal gradient were transferred for incubation at other temperatures, methane production was greatest at 65-70°C (Figure 2). Similar results were observed in the Wiegert Channel (data not shown). Incubation at 75°C and 80°C inhibited methane production in all samples. Although some methane production was indicated for the 50°C samples incubated at 75°and 80°C, this was most likely a result of methane production that occurred before samples could be transferred to higher incubation temperatures (i.e. during transit). Time courses of methane production showed no increases in methane production for samples incubated at 75°and 80°C. Incubation for several days, a period presumably sufficient to deplete immediate methane precursors, did not alter the results. Thus,anaerobic processes involved in the conversion of algal-bacterial material to methane appear to be most active at 65°-70°C and to have an upper temperature limit between 70° and 75°C.

Correlation of Primary Productivity and Methanogenesis

Primary production was sharply limited at temperatures above 50° to 55°C (Figure 3). In this experiment, measurements of methanogenesis and primary production were made on the same date in Octopus Spring. There was a reasonable correlation between primary productivity and methane production, both of which showed significant activity at only 50° and 55°C.

Isolation and Characterization of Methanogenic Bacteria

Attempts to isolate thermophilic methanogenic bacteria in pure culture failed at first as it was impossible to separate methane-producing bacteria from contaminant bacteria. A heterotrophic contaminant was readily isolated by dilution to extinction in BM + TYE. When BM was supplemented with 0.1 ml of an autoclaved, filtered turbid culture of the isolated heterotrophic bacterium grown in BM + TYE (BM + S), it was possible to obtain pure cultures of methanogenic bacteria at 50°, 55°, 60° and 65°C using Octopus Spring mat samples from these temperatures as sources of inocula. It was later found that vitamin B_{12} replaced the requirement for the growth factor supplied by the heterotrophic associate.

All methanogenic isolates were long, irregular, Gram positive, rod shaped bacteria which exhibited a blue-green autofluorescence. Hydrogen, but not formate, acetate or methanol served as an energy source for growth. All methanogenic isolates showed greatest growth and methane production at 65°C (Figure 4). Methane production and growth occurred when all isolates, except for the 50°C strain, were incubated at 70°C. No growth or methane production was observed at 75° or 80°C.

DISCUSSION

Ward (17) reported that maximum methane production in the Octopus Spring algal-bacterial mat occurred from 45°-55°C. Methane production was maximal between 50° and 60°C in the five springs studied here. It was surprising that maximum methane production was restricted to temperatures well below the upper temperature limit for mat development because incubation of samples at elevated temperatures increased methane production. Natural populations present in samples collected from different temperatures (50°-60°C) all showed maximum methanogenesis at higher temperatures. Similar results were observed upon incubation for a period presumed to be sufficient to exhaust immediate methane precursors (several days). Thus, other members of the anaerobic food chain presumably had similar temperature relations or methanogenic bacteria would have become substrate limited.

In order to study the temperature relations of methanogenic bacteria, isolates were obtained at 50°, 55°, 60° and 65°C. Except for the vitamin B_{12} requirement, these isolates appear to be similar to _M_. _thermoautotrophicum_ (23) which was previously isolated from Octopus Spring (21). All isolates showed optimal growth and methanogenesis at 65°C which was consistant with the results of temperature transfer experiments using natural algal-bacterial mat material. Although other microorganisms and processes in the natural thermal environment of algal-bacterial mats have been found to be adapted to the temperatures at which they are found (1,5,14), a single temperature strain of a H_2-utilizing methanogenic bacterium appears to exist at all temperatures in the Octopus Spring mat.

Since the bacteria involved in decomposition to methane in this system prefer to grow at 65°-70°C, some factor other than temperature must account for the fact that maximum methanogenesis occurs below the temperature the bacteria prefer. Doemel and Brock (9) and Ward (17) suggested that decomposition may be most active at temperatures where the production of organic matter is most active. The correlation between primary production and methanogenesis (Figure 3) suggests that methanogenesis may be tightly coupled to the supply of organic matter, the amount of which is related to the rate of mat formation.

Of obvious interest is the potential application of the natural thermophilic microflora to high temperature anaerobic conversions. Acclimation of mesophilic fermentations to temperatures above 60°C was found to be difficult (6,8,16). Although maximum decomposition of the mat to methane was restricted to 50°-60°C for ecological reasons, the microflora involved were most active at 65°-70°C. However, the application of natural thermophilic microflora to conventional thermophilic waste conversion processes may also be difficult. The basis for this statement is i) the observation that acetate consumption is primarily mediated by phototrophic microorganisms rather than by acetate-using methanogenic bacteria in the algal-bacterial mats (Sandbeck and Ward, submitted for publication) and ii) the well known association between accumulation of volatile fatty acids and digestor failure (20). The only thermophilic acetate-using methanogenic bacterium which has been described was only able to grow below 60°C (24).

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FIGURE LEGENDS

Figure 1. Temperature distribution of methanogenesis in hot spring algal-bacterial mats (methane produced after 17-20 hours).

Figure 2. Effect of temperature on methanogenesis in Octopus Spring algal-bacterial mat samples collected at various temperatures (methane produced after 15 hours).

Figure 3. Temperature distribution of primary production (○) and methanogenesis (●) in the Octopus Spring algal-bacterial mat (methane production after 9 hours; primary production, 1 1/2 hour incubation).

Figure 4. Growth of (○) and methanogenesis by (●) methane-producing bacteria isolated from Octopus Spring (measured at 72 hours). Isolates were obtained at 50°, 55°, 60° and 65°C (indicated in each box) and incubated at the temperatures indicated by the abscissa.

Fig. 1

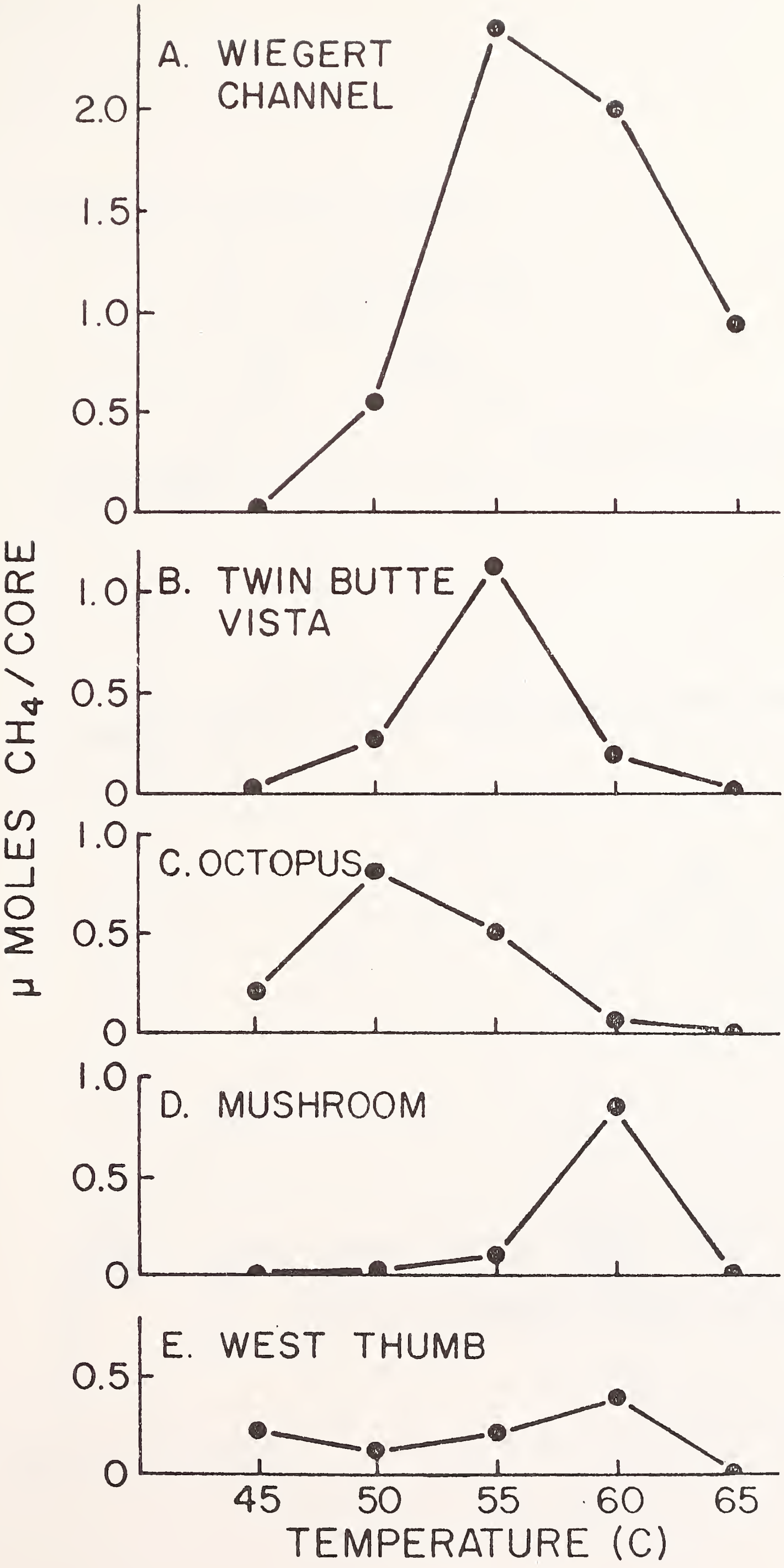

A. WIEGERT CHANNEL
B. TWIN BUTTE VISTA
C. OCTOPUS
D. MUSHROOM
E. WEST THUMB
μ MOLES CH_4 / CORE
0
0.5
1.0
1.5
2.0
45
50
55
60
65
TEMPERATURE (C)

Fig 2

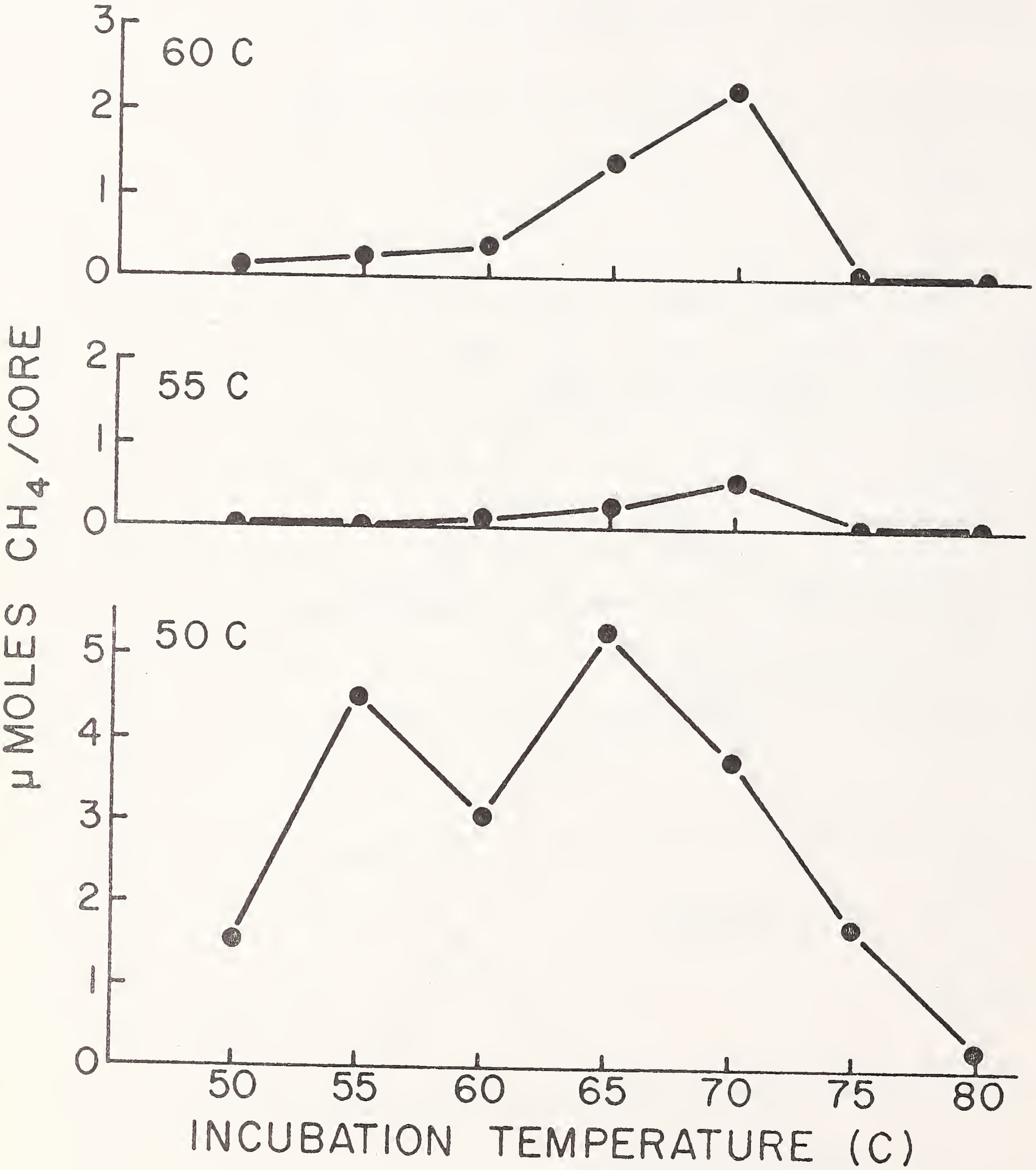

60 C
55 C
50 C
μMOLES CH4 /CORE
0
1
2
3
0
1
2
0
1
2
3
4
5
50
55
60
65
70
75
80
INCUBATION TEMPERATURE (C)

Fig 3

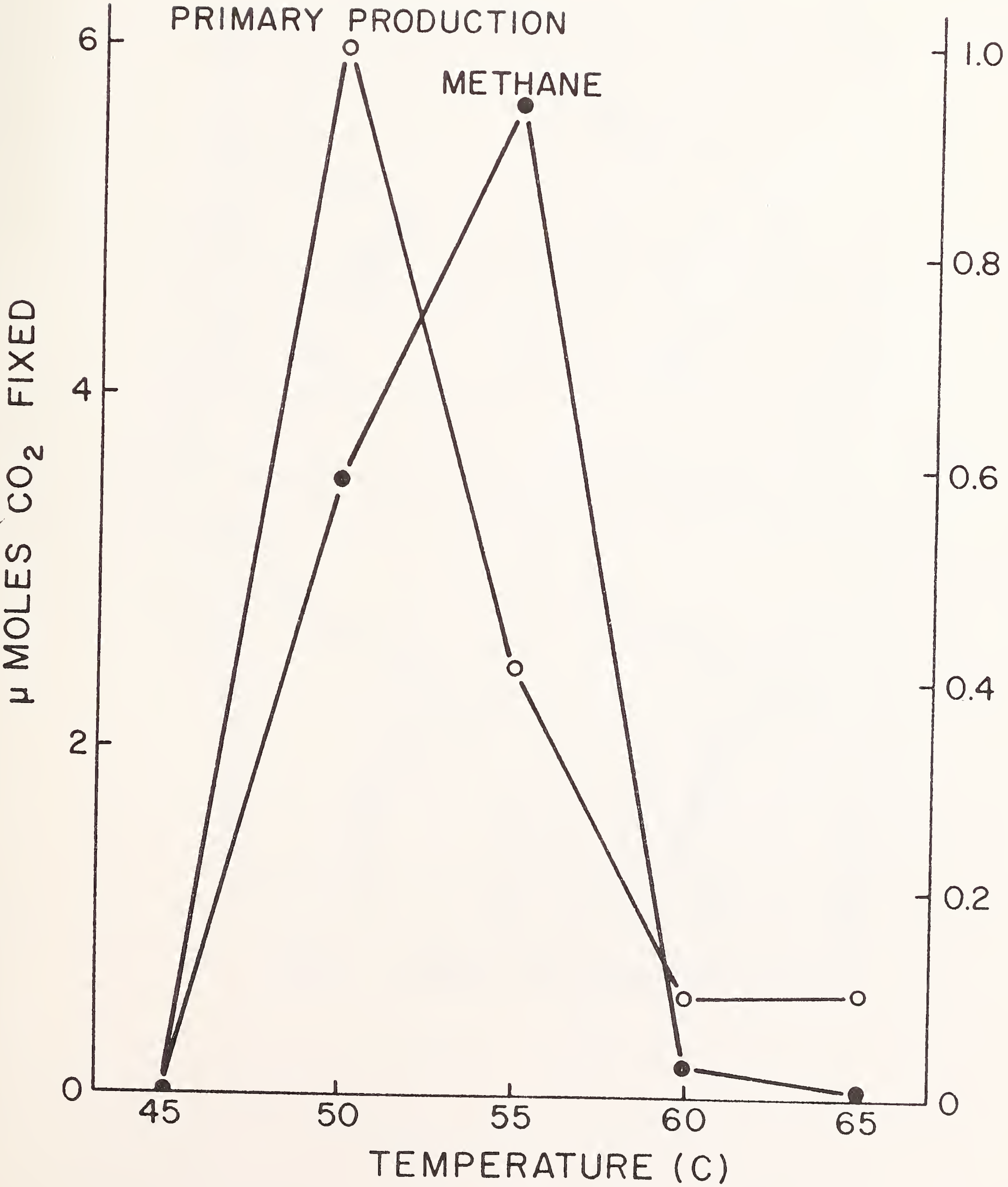
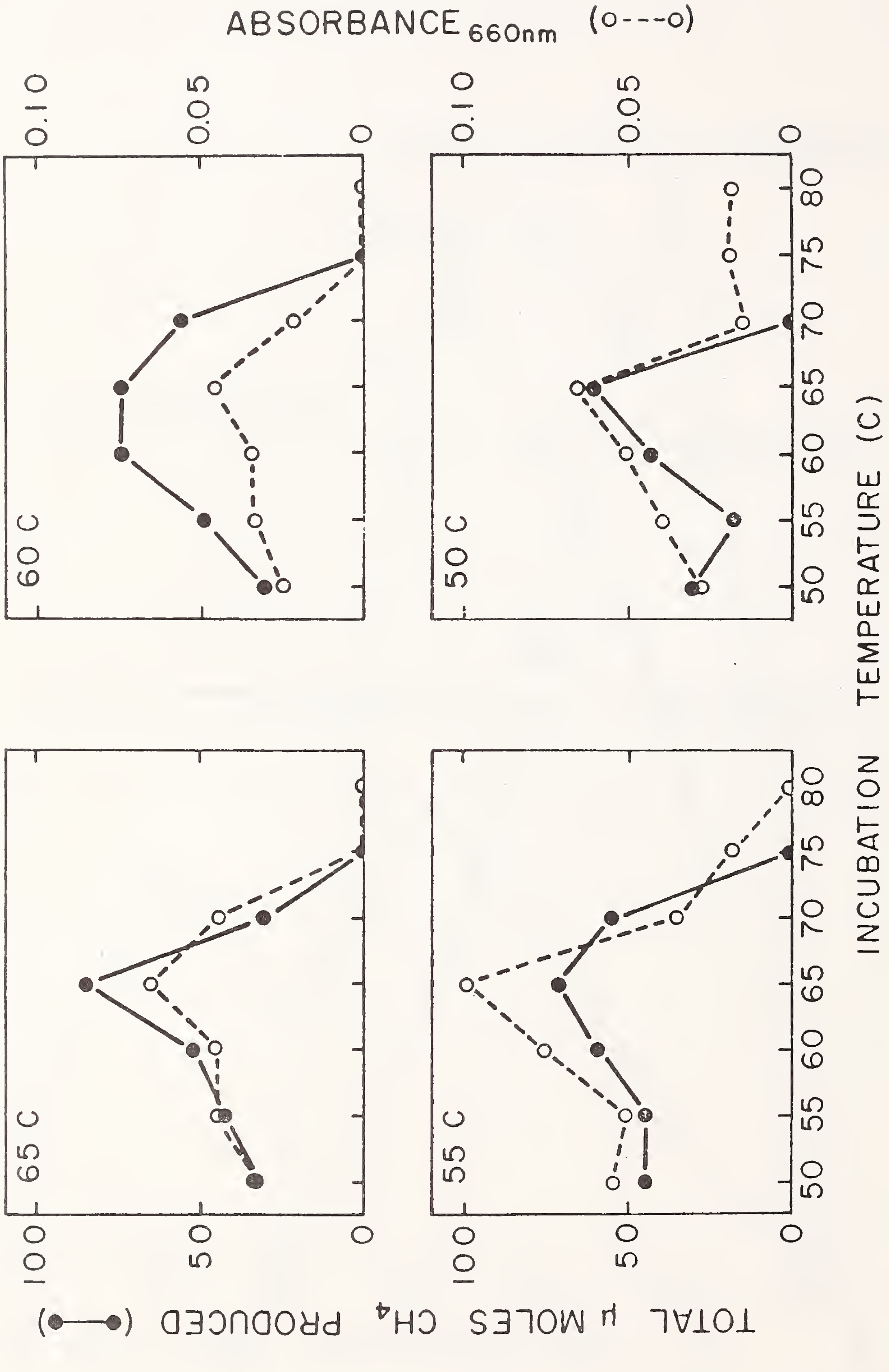

PRIMARY PRODUCTION
METHANE
μ MOLES CO_2 FIXED
μ MOLES CH_4/CORE
TEMPERATURE (C)
0
2
4
6
0
0.2
0.4
0.6
0.8
1.0
45
50
55
60
65

Fig 4


ABSORBANCE 660nm (o---o)
TOTAL μ MOLES CH_4 PRODUCED (●—●)
INCUBATION TEMPERATURE (C)
65 C
60 C
55 C
50 C
0.10
0.05
0
100
50
0
50
55
60
65
70
75
80

Appendix C. Submitted to Applied and Environmental Microbiology

FATE OF IMMEDIATE METHANE PRECURSORS IN LOW SULFATE HOT SPRING ALGAL-BACTERIAL MATS

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Running Head - Methane precursors in algal-bacterial mats

ABSTRACT

Experiments designed to determine the relative importance of methane precursors in algal-bacterial mats present in the effluent channels of low sulfate hot springs indicated that acetate was not a significant precursor of methane at either high or low concentration. At high acetate concentrations, [2-^{14}C]-acetate was incorporated into cellular material. Dark incubation reduced the amount of [2-^{14}C]-acetate incorporated. Autoradiograms prepared from mat material incubated with [2-^{14}C]-acetate showed that acetate was incorporated into very long filamentous bacteria. Experiments with $NaH^{14}CO_3$ showed that radioactive methane was produced rapidly from $H^{14}CO_3^-$ and that HCO_3^- reduction accounted for at least 70-80% of the methane evolved from algal-bacterial mat samples. Apparently, CO_2 is the main precursor of methane because acetate is incorporated by other inhabitants of this microbial community, possibly photoheterotrophic bacteria.

INTRODUCTION

Hot spring algal-bacterial mats represent a natural environment where thermophilic anaerobic decomposition of organic matter may be studied. Algal mats of alkaline, siliceous hot springs have also been suggested as modern analogs of the environments in which siliceous Precambrian stromatolites once formed (28, see 7). Thus, studies of their formation and decomposition may aid in interpretation of early life forms preserved as microfossils. Previous studies indicated the complete decomposition of algal-bacterial organic matter (7), but detailed studies of anaerobic decomposition are lacking (4,36). A complete anaerobic food chain was suggested by the observation of methane production (29) and sulfate reduction (30) as terminal anaerobic processes in algal-bacterial mats of low and high sulfate hot springs, respectively. This study was directed at determining the importance of immediate methane precursors in low sulfate hot spring algal-bacterial mats.

Acetate and carbon dioxide have been shown to be the major immediate methane precursors in many anaerobic environments (see 17,35). The relative importance of these methane precursors can be compared because acetate conversion to methane occurs by reduction of the intact methyl group and not by CO_2 as an intermediate (see 17, and 25,26,32). In different environments, the relative amounts of methane formed from these substrates varies (see 17 and 14,19,34). Acetate has been shown to account for 73-90% of the methane produced in anaerobic waste digestors, 60% of the methane formed in rice paddy soil, and 70% of the methane formed in lake sediments. Carbon dioxide has been shown to account for 14-30% of the methane formed in anaerobic waste digestors, 20-30% of the methane formed in rice paddies and 36-98% of the methane formed in lake sediments. In contrast to the usual importance of acetate in methanogenesis, only 5.3% of the methane formed in the rumen was derived from acetate (21), presumably because of its uptake by the animal.

In this paper we report that CO_2 is the major source of methane in hot spring algal-bacterial mats. Uptake by other inhabitants of this microbial community, possibly photoheterotrophs, may preclude acetate as a major methane precursor.

MATERIALS AND METHODS

Study Areas

The major research area used in this study was Octopus Spring, an alkaline hot spring (pH 8.0) located about 0.15 km SSE of Great Fountain Geyser in the White Creek drainage in Yellowstone National Park. At this spring, experiments were undertaken only in the southernmost effluent channel. Another research area used in this study was in a meadow, also in the Lower Geyser Basin of Yellowstone National Park, adjacent to Firehole Lake Drive. Springs in this meadow were collectively referred to as Serendipity Springs because of their chance discovery in early 1968 by M.L. and T.D. Brock (8). The study area in the Serendipity Springs group was an artificial effluent channel constructed by Fraleigh and Wiegert (8) by diverting the effluent of a spring so that it constantly flowed down a plywood channel (1.2 m wide x 24 m long). The pH of the piped in water ranged from 6.0 to 7.0 (8).

Some experiments were performed only in the 50-55°C region where maximum methanogenesis occurred (Sandbeck and Ward, submitted for publication).

Sampling

Whole cores were removed from the algal-bacterial mat with a no. 4 brass cork borer (50.3 mm^2) and transferred directly to one dram glass vials (Kimble, 14.5 x 45 mm) which were sealed anaerobically (13, except that no copper reducing column was used in the field) under a stream of 100% helium (Linde). Recessed butyl rubber stoppers (A.H. Thomas, size 00) were used to effect a seal and keep the vials anaerobic during later manipulations. Stoppers were secured by lengthwise taping with masking tape, except for "light" incubation where tape was fixed only at the vial neck, and "dark" incubation where vials were additionally wrapped in several layers of aluminum foil. Anaerobically tubed samples were either incubated in the effluent channel or quickly placed in insulated coolers that contained water which was 5°C warmer than the in situ temperature. During transit to the laboratory (approximately 2 hours), samples cooled slightly, but this procedure ensured that samples would be kept within 5°C of their indigenous temperatures. In the laboratory, samples were transferred to dark incubators that matched the in situ temperature. All additions were made from anoxic sterile stock solutions using a 1 ml disposable syringe (Plastipak syringe, Becton-Dickinson) at the time of sample collection unless otherwise stated.

Fate of [2-^{14}C]-acetate

Replicate vials (2-4) received 0.1 ml of a 10 μCi/ml stock solution of [2-^{14}C]-acetate (sodium salt, 44 mCi/mmol, New England Nuclear). Unless otherwise stated, biological activity was terminated after two hours by the addition of 0.5 ml formalin accompanied by extremely vigorous shaking to ensure that the formalin permeated the gelatinous sample. Gas headspace subsamples were removed for analysis of $^{14}CO_2$, CO_2, $^{14}CH_4$ and CH_4 as described below. The pH (VWR pH Master pH meter plus glass combination electrode), and headspace and liquid volumes (determined by displacement with water) were measured after removal of samples so that results could be corrected to a per core basis (see below). Incorporated radioactivity was determined by filtering a 0.1 ml aliquot of a homogenized sample (teflon tissue homogenizer) diluted with 0.9 ml distilled water through a 0.45 µm membrane filter (Millipore). After the filtrate had been collected, the filter was rinsed with 0.5 to 1.0 ml distilled water, dried and exposed to concentrated HCl fumes overnight to remove ^{14}C-carbonates. Radioactivity in filters and in 0.1 ml of the filtrate was determined in 10 ml Aquasol (New England Nuclear) on a model LS 100-C liquid scintillation counter (Beckman) using the ^{3}H + ^{14}C window and a gain of 240. Correction for differences in counting efficiency were made by the automatic external standard method. Data were corrected to a per core basis for comparison with radioactivity in other fractions.

Fate of [^{3}H]-acetate

Replicate vials received 0.1 ml of a 20 µCi/ml stock solution of [^{3}H]-acetate (sodium salt, 2 Ci/mmol, New England Nuclear). Vials were poisoned with formalin after 3 hours incubation in the effluent channel or 4 days incubation in a laboratory incubator. Gaseous subsamples were analyzed for $C^{3}H_4$ as described in the analytical methods section. Incorporated and unincorporated radioactivity were determined as described above except that the ^{3}H window and a gain of 270 were used.

Conversion of $NaH^{14}CO_3$ to $^{14}CH_4$

Replicate vials received 0.1 ml of a 20 µCi/ml stock solution (pH 8.0) of $NaH^{14}CO_3$ (44.5 mCi/mmol, New England Nuclear). CH_4, $^{14}CH_4$, CO_2 and $^{14}CO_2$ were followed over time as described in the analytical methods section. Methane (nmoles) produced from CO_2 was obtained by dividing dpm $^{14}CH_4$/core by the specific activity of CO_2 (dpm/nmole).

The relative importance of CO_2 as a methane precursor was determined by calculating sp act CH_4/sp act CO_2 (14). In some experiments hydrogen (0.1 ml 100% H_2 (Linde) or sodium acetate (0.1 ml to achieve a final concentration of 1 mM in an estimated 2.5 ml sample) were added as described in the Results section to enhance different methane formation reactions (see 34). In some cases, vials were flushed with O_2-free helium (Linde) before determination of the specific activity ratio.

Isotopic selection of ^{12}C over ^{14}C in $NaH^{14}CO_3$ reduction to $^{14}CH_4$ or the error in measurement of sp act CH_4/sp act CO_2 was checked by similar additions of $NaH^{14}CO_3$ to pure cultures of H_2-utilizing thermophilic methanogenic bacteria resembling Methanobacterium thermoautotrophicum isolated at 50°, 55°, 60° and 65°C from the Octopus Spring algal-bacterial mat. Culture conditions were as described by Sandbeck and Ward (submitted for publication) except that the gas phase was 100% H_2. Incubation was at the temperature at which each isolate was obtained. Determination of sp act CH_4/sp act CO_2 was done after 24 hours, before significant depeletion of CO_2.

Analytical Methods

Gas samples were removed from the headspace of vials using a helium flushed glasspak syringe (Becton-Dickinson) attached to a mininert valve (Supelco) to minimize loss of sample due to pressure differences. Gas subsamples (0.2 ml) were analyzed for CH_4, $^{14}CH_4$, CO_2 and $^{14}CO_2$ by gas chromatography-gas proportion analysis using a method similar to that of Nelson and Zeikus (20). Details of the method were described by Ward and Olson (30) except that gas concentrations and radioactivity were quantified using a Spectra-Physics model 4100 computing integrator and a Spectra-Physics Minigrator, respectively. C^3H_4 was detected in the same way, but with the gas proportional counter furnace turned off to prevent combustion of methane. To ensure detection by this method, C^3H_4 was prepared by incubating 2 μCi of [^{3}H]-acetate with contents of an anaerobic dairy cow manure digestor until no further increases in C^3H_4 were detected. By comparing minimum and maximum responses to C^3H_4 it was calculated that no more than 0.29% of the [^{3}H]-acetate added to algal-bacterial mat samples could have been converted to C^3H_4 or it would have been detected. The total amounts of CH_4 and $^{14}CH_4$ per core were calculated by comparison of subvolume to the gas headspace volume. Total amounts of CO_2 and $^{14}CO_2$ per core were determined by correction for the difference between subsample and headspace volume, and also for gas solubility and dissociation equilibria according to Stainton (27).

Autoradiography and Microscopy

Autoradiograms of material incubated with [2-^{14}C]-acetate were prepared after the method of Brock and Brock (3). A thin film of homogenate was smeared onto a glass slide (precleaned glass slides - VWR Scientific) and allowed to air dry. The slides were put through a series of five distilled water rinses (one minute each) to remove any unincorporated radioactivity. Slides were then dipped for five seconds in photographic emulsion (Kodak NTB2) under a Kodak No. 2 safelight. Slides were exposed for 24-26 days in total darkness and then developed in total darkness with Kodak D-19 developer and fixed with Kodak fixer. Slides were examined using a Leitz Ortholux II microscope equipped for interference contrast optics. Photomicrographs were taken with a Nikon Microflex model EFM semi-automatic photomicrographic attachment at 500 X using Kodak Panatomic-X film. Exposure time was 1/2 - 1 second. Negatives were then enlarged on silver bromide print paper (Koda Bromide F-4) to achieve better contras

Autofluorescence was observed using the Leitz Ortholux II microscope which was also equipped with ultraviolet light vertical illumination from an HBO-200W mercury lamp. The ultraviolet light passed through a Leitz B cube excitation and emission filter combination.

RESULTS

Microscopic Observations

Surface samples from algal-bacterial mat cores were examined by fluorescence microscopy. The red autofluorescence of chlorophyll a in cyanobacteria suggested the predominance of organisms resembling Synechococcus lividus in both hot springs, and the presence of microorganisms resembling Mastigocladus laminosus and Phormidium sp. (4,5) as minor components of the Wiegert Channel mat. Samples which were incubated anaerobically and in which substantial methanogenesis had occurred, also showed the presence of rod shaped bacteria which exhibited a blue-green colored autofluorescence similar to the autofluorescence of thermophilic methanogenic bacteria isolated from the same mats. No blue-green fluorescing sarcina or pseudosarcina shaped cells were observed.

Fate of Acetate

Two hour incubation of algal-bacterial mat cores from Octopus Spring and the Wiegert Channel with [2-^{14}C]-acetate (22.7 nmoles added acetate) resulted in the production of small amounts of $^{14}CH_4$ and $^{14}CO_2$ (Table 1). Most of the label was taken up by cells, or was recovered in the filtrate (presumably as unmetabolized acetate). Except in one condition where 11.06% of the label was recovered as radioactive methane, all other conditions showed less than 1% of the label appearing as $^{14}CH_4$. Vials that were left completely exposed to sunlight had more label enter the cellular pool than those sample vials that were darkened by aluminum foil.

When samples were preincubated 48 hours in the dark prior to [2-^{14}C]-acetate addition, only 1.9-8.4% of the label was incorporated (Table 2). Even though substantial methane production occurred, incubation with [2-^{14}C]-acetate for 71 hours did not result in substantial $^{14}CH_4$ or $^{14}CO_2$ production. An exception was at 55°C in the Wiegert Channel where 39% of the label appeared as $^{14}CH_4$. Most of the label remained in solution.

Experiments with tritiated acetate were performed at 50°C in the Octopus Spring algal-bacterial mat to determine if the flow of label was similar to that observed in [2-^{14}C]-acetate experiments where much higher levels of acetate were added. Sample vials in this experiment received 2.0 μCi of [^{3}H]-acetate which was equivalent to adding 1 nmole acetate. Tritiated methane was not detected (Table 3) in either short (3 hours in the effluent channel) or long (4 days in a dark incubator) incubations. By means of a parallel experiment (see Materials and Methods) where the same amount of tritiated acetate was added to a sample of cattle waste digestor material, it was determined that no more than 0.29% of the added label could have appeared as methane or it would have been detected. Most of the label (97-98%) remained in the filtrate and the remainder (1-2%) was taken up by cells.

$H^{14}CO_3^-$ as a Methane Precursor

When $NaH^{14}CO_3$ was added to samples from Octopus Spring or Wiegert's Channel, rapid linear production of $^{14}CH_4$ ensued (Figure 1). The ratio sp act CH_4/sp act CO_2 indicated the fraction of methane derived from CO_2 reduction. The specific activity ratio increased from about 0.5 to about 0.8 over the initial 6 to 8 hours after addition of $NaH^{14}CO_3$ to algal-bacterial mat samples, but several observations suggested that the initial rise in the specific activity ratio was an artifact of the system rather than a reflection of a changing relative importance of methane production reactions. For example, Figure 2 shows that the specific activity ratio change also occurred if label was added well after sampling (46 hours) indicating that the change was related to addition of $NaH^{14}CO_3$ and not to time after sampling. Also, additions of H_2 and acetate (to artificially enhance the importance of different methane production reactions) did not markedly alter the specific activity ratio as would be expected if other methane production reactions were occurring (Table 4).

To ensure that specific activity comparisons were made after initial changes in the specific activity ratio, the headspace of each vial was flushed with helium 24 hours after $NaH^{14}CO_3$ addition and reincubated 3 hours before determination of the specific activity ratio. Specific activity ratios determined in this way were similar to those determined 6-8 hours following $NaH^{14}CO_3$ addition (see Table 4). This procedure was adopted in subsequent specific activity ratio determinations.

When data from different experiments were pooled, mean specific activity ratios of 0.80 (± 0.10 standard deviation, n=10) for Octopus Spring (55°C) and 0.71 (± 0.24 standard deviation, n=5) for Wiegert's Channel (60°C) indicated that 71-80% of the methane formed was by reduction of CO_2. Similar results indicating the predominance of CO_2 as a methane precursor were noted at all temperatures in Octopus Spring (Table 5). Specific activity ratios of 0.84, 0.81, 0.83 and 0.83 were found for pure cultures of thermophilic methanogenic bacteria isolated and grown at 50°, 55°, 60° and 65°C, respectively.

Autoradiograms

Autoradiograms prepared from [2-^{14}C]-acetate labelled material obtained from Octopus Spring (55°C) are shown in Figure 3. The label was taken up by very long, filamentous microorganisms. Similar results were noted for [2-^{14}C]-acetate labelled material obtained at other temperatures in Octopus Spring and the Wiegert Channel.

DISCUSSION

Methanogenic bacteria have been shown to use H_2, acetate, formate, methanol, carbon monoxide and various methyl amines as energy sources (1). However, in natural environments, the predominant methane precursors are thought to be H_2/CO_2 and acetate (17,35). Since CO_2 reduction and acetate conversion to methane occur by different mechanisms, $NaH^{14}CO_3$, [2-^{14}C]-acetate and [^{3}H]-acetate were used to selectively study the contribution of either methane production reaction. When [2-^{14}C]-acetate was added to sample vials, very little $^{14}CH_4$ was formed at any temperature in Octopus Spring or Wiegert Channel algal-bacterial mats (Table 1). Even when samples were incubated with [2-^{14}C]-acetate for 71 hours, in a period of substantial methane production, little $^{14}CH_4$ was produced. These results indicate that acetate is not a significant source of methane in this environment. Small amounts of $^{14}CO_2$ produced from [2-^{14}C]-acetate (Tables1 and 2) could be explained by anaerobic acetate oxidation by sulfate-reducing bacteria (33). Sulfate reduction has been previously reported in the Octopus Spring algal-bacterial mat (6), and rapid reduction of $^{35}SO_4^=$ to $H_2{}^{35}S$ also occurred in the Octopus Spring and Wiegert's Channel mats (Mike Winfrey - personal communication). The $^{14}CH_4$ detected in [2-^{14}C]-acetate labelling experiments may have come from reduction of $^{14}CO_2$ rather than by direct reduction of the methyl carbon of acetate. This possibility is especially likely in the experiment where [2-^{14}C]-acetate was added 48 hours after sampling and samples were not assayed until 71 hours after label addition.

In short term labelling experiments with [2-^{14}C]-acetate, it was noted that ^{14}C was rapidly incorporated into cellular material. This phenomenon was observed at all temperatures in both Octopus Spring and Wiegert's Channel. The incorporation of [2-^{14}C]-acetate into cellular carbon was decreased by dark incubation (and by addition of label after two days of incubation in the dark, see Table 2) which suggests that phototrophic microorganisms may be involved in acetate incorporation. Autoradiograms of [2-^{14}C]-acetate labelled algal-bacterial mat material from Octopus Spring and Wiegert's Channel were prepared so that incorporated ^{14}C could be related to the type of cell involved in acetate incorporation. Representative results from Octopus Spring (55°C) (Figure 3) clearly indicated that very long filamentous bacteria incorporated most of the ^{14}C into cellular material. It is reasonable to guess that acetate was incorporated by the filamentous phototrophic bacterium Chloroflexus aurantiacus because i) this organism is common to both of the algal-bacterial mats sampled, ii) light/dark experiments indicated the involvement of a phototrophic microorganisms and iii) the ability of C. aurantiacus to grow photoheterotrophically using acetate as a carbon source has been shown (16,23,24). Pierson (B.K. Pierson, Ph.D. thesis, Oregon State University, Eugene, 1973) and Bauld and Brock (2) were led to the same conclusion when these workers also noticed incorporation of radiolabelled acetate into Chloroflexus-like filaments in hot spring algal mats.

It was considered a possibility that an increase in the acetate pool upon addition of [2-^{14}C]-acetate (to approximately 11.5 μM) might alter the flow of acetate away from reactions such as conversion to methane. A reaction unimportant at low acetate concentration (such as uptake by photoheterotrophic cells) might be enhanced at artificially high acetate pool levels. By using [^{3}H]-acetate it was possible to study the fate of acetate at a lower added acetate concentration (to approximately 0.5 μM). Results (Table 3) indicated that acetate was less rapidly incorporated at the lower acetate concentration. This might reflect the concentration dependence for acetate incorporation. No C^3H_4 was detected even after incubation for 4 days indicating that acetate conversion to methane was not a reaction favored by low acetate concentrations.

$NaH^{14}CO_3$ labelling experiments indicated that 70-80% of the methane formed at all temperatures was derived from CO_2, which was consistent with the lack of importance of acetate as a major methane precursor. Over the initial 6-8 hours after the addition of $NaH^{14}CO_3$ to algal-bacterial mat samples, change in sp act CH_4/sp act CO_2 probably was not a reflection of a changing relative importance of methane production reactions as i) the phenomenon was related to the addition of radiolabel and was not related to time after sampling and ii) addition of H_2 or acetate did not significantly alter the specific activity ratio (Table 4). The addition of these methane precursors markedly changed the relative importance of methanogenic reactions in lake sediments (34) and in a dairy cow manure digestor (unpublished results of this laboratory) where both acetate and CO_2 are known to be important methane precursors. The lack of change in the relative importance of CO_2 reduction to methane following acetate addition was further evidence that the conversion of acetate to methane was not significant in algal-bacterial mat samples. It was assumed that the change in the specific activity ratio was an artifact related to the dispersal of the added $NaH^{14}CO_3$ to methanogenic bacteria residing within the compact, gelatinous sample.

Despite the high relative importance of CO_2 in methanogenesis, specific activity ratios were always less than 1.0 (which indicates 100% of the methane formed is derived from CO_2 reduction). Slight increases in the specific activity ratio upon addition of H_2 could indicate the contribution of other known methane precursors such as methylamines (11,22,31,39) or methyl sulfides (37) to the formation of methane. Zinder, et al. (33) showed the formation of methyl sulfides in the degradation of Octopus Spring algal-bacterial mats. Methane-producing bacteria are also known to carry out one of the greatest biological carbon isotope discriminations known (see 17). In an experiment to determine the specific activity ratio in pure cultures of isolated methanogenic bacteria, the highest sp act CH_4/sp act CO_2 found was 0.84 suggesting an apparent isotopic discrimination between ^{12}C and ^{14}C of about 16%. A preference of ^{12}C over ^{13}C of 2.5-3.4% has been reported during CO_2 reduction to CH_4 by M. thermoautotrophicum (9,10). As the mass difference between ^{12}C and ^{14}C is twice the mass difference between ^{12}C and ^{13}C, the discrimination between ^{12}C and ^{14}C should be twice as great as between ^{12}C and ^{13}C (15). Thus, up to 6.8% of the specific activity dilution between $^{14}CH_4$ and $^{14}CO_2$ could be explained by a preference for the lighter nonradioactive ^{12}C. The difference between the apparent discrimination value in pure cultures and the highest reported fractionation value could be due to error in the four determinations needed to calculate the specific activity ratio. The apparent discrimination in pure cultures is sufficient to correct specific activity ratios determined in field work to near 1.0 suggesting that CO_2 reduction may account for nearly all of the methane produced in the anaerobic degradation of hot spring algal-bacterial mats.

High temperature is not a barrier to microbial conversion of acetate to methane as Zinder and Mah (39) reported the isolation of a thermophilic strain of Methanosarcina capable of growth on acetate at 55°C. Blue-green autofluorescence of methanogenic bacteria has been suggested as presumtive evidence for their recognition in natural materials (18). Only rod shaped blue-green autofluorescing cells were observed in the decomposing mat samples. In the Octopus Spring mat, the photoheterotrophic C. aurantiacus is active within the entire 1-3 mm photic zone because of its ability to grow at low light intensity beneath the cyanophyte S. lividus which requires higher light intensity (2,4,7). Maximum rates of methanogenesis were also reported to be in the 2-4 mm depth intervals in Octopus Spring (29). The proximity of active photoheterotrophic microorganisms and anaerobic processes may lead to acetate consumption by photoheterotrophic bacteria rather than by methanogenic bacteria. It has also been suggested that acetate may not be an important energy source for sulfate-reducing bacteria in a high sulfate hot spring algal-bacterial mat, where acetate accumulates upon dark incubation even during active sulfate reduction (30). A similar situation exists in the rumen and gastrointestinal fermentations where methane is produced mainly from CO_2 because the host animal absorbs acetate for its energy metabolism (12,17).

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Table 1. Fate of [2-^{14}C]-acetate incubated anaerobically for 2 hours in hot spring algal-bacterial mat cores.

CONDITION	% OF RECOVERED ^{14}C IN: CO_2	CH_4	CELLS	FILTRATE
A. OCTOPUS SPRING				
45 C	1.59	-	74.08	24.34
50 C	2.53	-	58.86	38.61
55 C LIGHT	1.32	0.13	44.71	53.85
55 C DARK	0.58	0.91	25.79	72.74
60 C	2.07	0.04	66.21	31.69
65 C	5.20	-	66.99	24.81
B. WIEGERT CHANNEL				
45 C	0.98	-	64.01	35.01
50 C	0.82	-	71.45	27.73
55 C LIGHT	1.01	0.32	86.10	12.57
55 C DARK	4.15	11.06	52.81	31.99
60 C	1.09	0.07	85.24	13.60

Table 2. Fate of [2-^{14}C]-acetate added to hot spring algal-bacterial mat cores 48 hours after collection and anaerobic incubation in the dark.

CONDITION	% OF RECOVERED ^{14}C IN:[a]			
	CO_2	CH_4	CELLS	FILTRATE
A. OCTOPUS SPRING				
50 C	0.39	0.12	4.39	95.11
55 C	0.29	5.44	3.70	90.27
60 C	0.36	0.20	6.64	92.82
B. WIEGERT CHANNEL				
50 C	0.07	0.13	2.67	97.16
55 C	1.24	38.94	8.38	53.54
60 C	0.18	0.11	1.93	97.79

[a] Samples assayed 71 hours after label addition.

Table 3. Fate of [^{3}H]-acetate incubated anaerobically in Octopus Spring algal-bacterial mat cores collected at 50°C.

INCUBATION TIME	% OF RECOVERED ^{3}H IN:		
	CH_4	CELLS	FILTRATE
3 HOURS	ND[a]	1.23	98.75
4 DAYS	ND	2.22	97.75

[a] Not detectable.

Table 4. Influence of hydrogen or acetate on the relative importance of CO_2 as a methane precursor in Octopus Sp. algal bacterial mat samples collected and incubated anaerobically at 55°C.

CONDITION	sp act CH_4/sp act CO_2 AT HOUR AFTER SAMPLING[a] 6	27[b]
CONTROL	0.69	0.73
HYDROGEN[c]	0.90	0.86
ACETATE[d]	0.84	0.86

[a] Specific activity CH_4 ÷ specific activity CO_2 x 100 = % CH_4 from CO_2.

[b] Headspace flushed at 24 hours with 100% helium (5 minutes).

[c] 0.1 ml 100% H_2 added at the time of sampling and again immediately after flushing with helium.

[d] 0.1 ml sterile anoxic Na acetate to achieve a final concentration of 1 mM (assuming a 2.5 ml sample) added at the time of sampling and immediately after flushing with helium.

Table 5. Relative importance of CO_2 as a methane precursor at various temperatures of the Octopus Spring algal-bacterial mat.

TEMPERATURE	sp act CH_4/sp act CO_2[a,b]
45 C	0.81
50 C	0.87
55 C	0.88
60 C	0.90

[a] Specific activity CH_4 ÷ specific activity CO_2 x 100 = % CH_4 from CO_2.

[b] Readings taken 3 hours after headspace flushed with 100% helium (5 minutes).

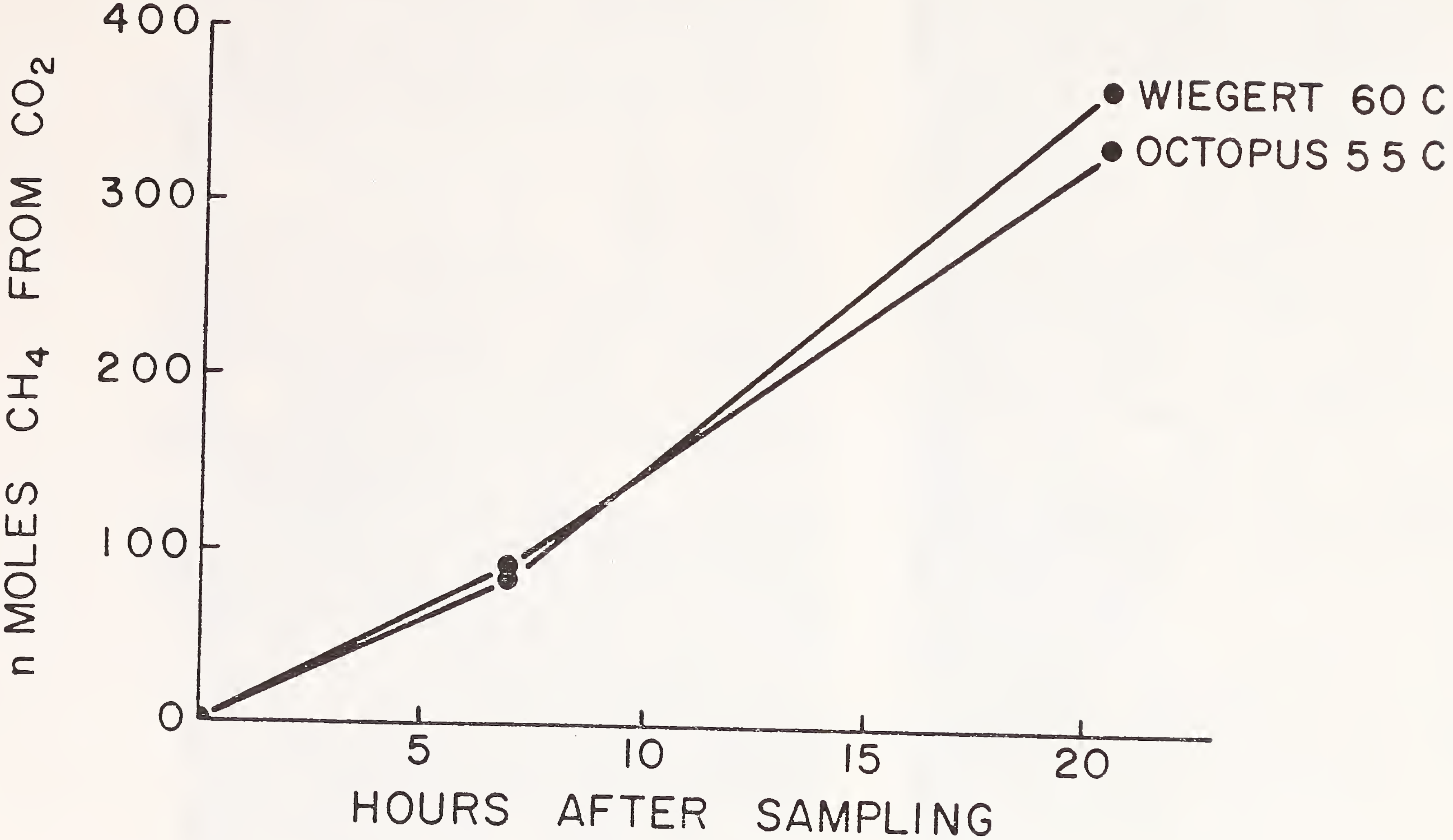
FIGURE LEGENDS

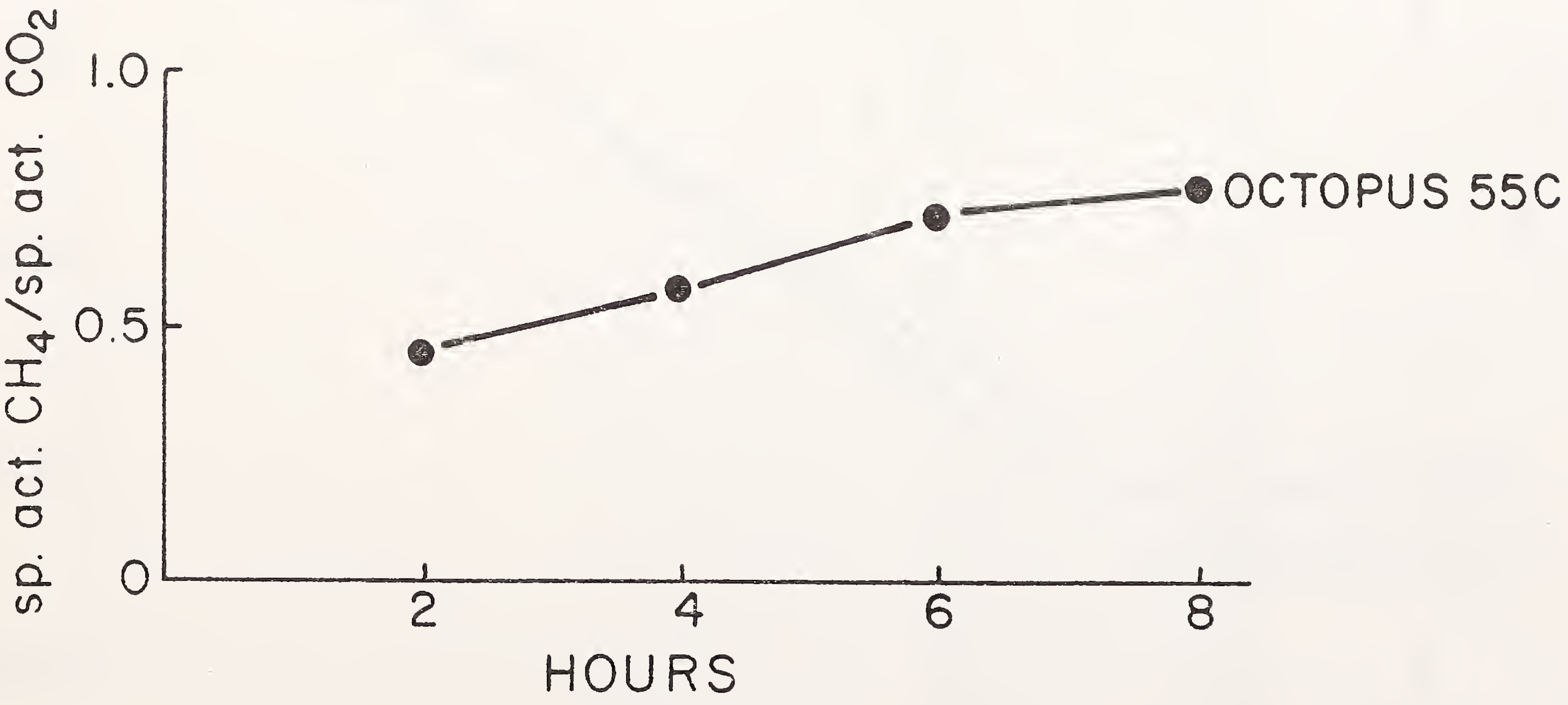
Figure 1. Production of methane from CO_2 in hot spring algal-bacterial mat cores.

Figure 2. Change in sp act CH_4/sp act CO_2 following addition of $NaH^{14}CO_3$ to algal-bacterial mat cores 46 hours after collection and anaerobic incubation in the dark.

Figure 3. Autoradiograms of Octopus Spring algal-bacterial mat samples collected and incubated anaerobically for 2 hours at 55°C with [2-^{14}C]-acetate. Magnification 1280x.

Fig 1


400
300
200
100
0
n MOLES CH_4 FROM CO_2
5
10
15
20
HOURS AFTER SAMPLING
WIEGERT 60 C
OCTOPUS 55 C


sp. act. CH4/sp. act. CO2
1.0
0.5
0
2
4
6
8
HOURS
OCTOPUS 55C

402782

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Terminal Processes in the Anaerobic Degradation of an Algal-Bacterial Mat in a High-Sulfate Hot Spring

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The algal-bacterial mat of a high-sulfate hot spring (Bath Lake) provided an environment in which to compare terminal processes involved in anaerobic decomposition. Sulfate reduction was found to dominate methane production, as indicated by comparison of initial electron flow through the two processes, rapid conversion of $[2\text{-}^{14}C]$acetate to $^{14}CO_2$ and not to $^{14}CH_4$, and the lack of rapid reduction of $NaH^{14}CO_3$ to $^{14}CH_4$. Sulfate reduction was the dominant process at all depth intervals, but a marked decrease of sulfate reduction and sulfate-reducing bacteria was observed with depth. Concurrent methanogenesis was indicated by the presence of viable methanogenic bacteria and very low but detectable rates of methane production. A marked increased in methane production was observed after sulfate depletion despite high concentrations of sulfide (>1.25 mM), indicating that methanogenesis was not inhibited by sulfide in the natural environment. Although a sulfate minimum and sulfide maximum occurred in the region of maximal sulfate reduction, the absence of sulfate depletion in interstitial water suggests that methanogenesis is always severely limited in Bath Lake sediments. Low initial methanogenesis was not due to anaerobic methane oxidation.

The interaction between sulfate-reducing and methanogenic bacteria has recently been described for low-sulfate freshwater sediments where methane production is the predominant terminal process in the anaerobic decomposition of organic matter (3–5, 37). In Lake Mendota sediments, the inhibition of methane production by added sulfate was overcome by addition of excess hydrogen or acetate (37). Thus, the basis for sulfate inhibition of methane production appears to be competition for common energy sources. The recent discovery of acetate-utilizing, sulfate-reducing bacteria (36) provided further evidence that both methane precursors could also serve as electron donors for sulfate-reducing bacteria.

The mutual exclusion of methane production and sulfate reduction is suggested by the mutual exclusion of methane and sulfate with depth in marine sediments (20, 22, 29). Variation in methanogenesis in salt marsh sediments has also been related inversely to sulfate concentration (18). However, methane production has not been well characterized in marine or other high-sulfate sediment systems (20). Although the requirement for sulfate depletion before methanogenesis was suggested on long-term incubation of marine (21) or brackish (26) sediments, concurrent methanogenesis and sulfate reduction were reported during incubation of nearshore subtidal sediment (27). Stimulation of methane production by addition of hydrogen to nearshore sediments (27) or hydrogen and carbon dioxide in salt marsh sediment (1) suggested that competition for common electron donors may limit methane production in marine sediments. Mountfort et al. (23) reported low rates of methanogenesis and predominant oxidation of $[2\text{-}^{14}C]$acetate to $^{14}CO_2$ (respiratory index = $^{14}CO_2/(^{14}CO_2 + {}^{14}CH_4)$ [see reference 38] was 0.97 to 0.99), indicating the predominance of sulfate reduction in intertidal sediments of Delaware Inlet, New Zealand.

In this study the terminal anaerobic processes sulfate reduction and methane production were studied in a high-sulfate hot spring where an algal-bacterial mat develops at the sediment-water interface. The spring under study, Bath Lake (Yellowstone National Park), has received attention because of the sulfide-adapted thermophilic, phototrophic community comprising the algal-bacterial mat (6). This community includes the cyanobacteria *Spirulina labyrinthiformis*, *Synechococcus lividus*, *Synechococcus minervae* and the photosynthetic flexibacterium *Chloroflexus aurantiacus* (6). The anaerobic degradation of algal-bacterial organic matter to methane was shown previously in a low-sulfate hot spring (35). The algal-bacterial mat of Bath Lake provided a natural high-sulfate environment where organic matter was decomposed anaerobically so that the interaction between methanogenic and sulfate-reducing bacteria could be compared.

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METHODS

Study area. Bath Lake is a neutral-pH thermal pool formed by several springs located in the upper terrace group at Mammoth in Yellowstone National Park. The precise location was given by Castenholz (6). The sampling area was along the southern edge near sources (50 to 55°C) where the temperature of water overlying a uniform algal-bacterial mat was always near 40°C.

Sampling and incubation. (i) Biological activity. Samples were removed by vertical coring with a brass cork borer (no. 4 [50.2 mm^2] for whole-core experiments, no. 6 [78.5 mm^2] for subcore experiments) to roller culture tubes (150 by 16 mm, Bellco) or 2-dram (ca. 7.4 ml) vials (16.75 by 60 mm, Acme Vial and Glass, Co.) which were capped with recessed butyl rubber stoppers (size 00, A. H. Thomas) under a continuous flow of helium in the field (after ref. 11). In some experiments, cores, were subsectioned before tubing in the field at approximate depth intervals. Anaerobically tubed samples were quickly placed in a styrofoam cooler containing warm water to keep samples within 5°C of the indigenous temperature during transit (approximately 2 h). In the laboratory samples were transferred to a 40°C incubator. All additions were made from anoxic stock solutions at the time of sample collection or immediately on return to the laboratory (sulfate reduction experiments only).

The following processes were assayed as described on anoxically tubed samples. For sulfate reduction, after addition of 0.2 ml of Formalin to duplicate samples (as abiological controls), replicate vials received 0.5 ml (0.2 μCi) of $Na_2{}^{35}SO_4$ (923 mCi/mmol, New England Nuclear) diluted in anoxic Bath Lake water. After 24 or 48 h of incubation, replicate vials were fixed by addition of 0.5 ml of 1 N zinc acetate followed by 0.2 ml of Formalin. Counts per minute of $H_2{}^{35}S$ were determined after distillation of samples acidified to less than pH 1 under N_2 into a series of three traps comprised of 0.2 ml of 2 N NaOH in 9 ml of Aquasol (New England Nuclear). Radioactivity was trapped in the first two traps, which were summed to represent total $H_2{}^{35}S$ per sample. Counts per minute of added $Na_2{}^{35}SO_4$ were determined in 10 ml of Aquasol. A model LS 100-C liquid scintillation counter (Beckman) was used to determine radioactivity. Sulfate was determined as described below. Correction for differences in counting efficiency were by the automatic external standard method. Sulfate reduction rates were calculated by the formula of Ivanov (12).

For the metabolism of ^{14}C-labeled compounds, replicate vials containing whole cores or subcores received either 0.25 ml (whole cores) or 0.5 ml (subcores) of 1-μCi/ml $NaH^{14}CO_3$ (7 mCi/mmol, New England Nuclear), 0.5 ml of 1-μCi/ml [2-^{14}C]acetate (54 mCi/mmol, New England Nuclear), or 0.1 ml of 33.3-μCi/ml $^{14}CH_4$ (gas, 30 mCi/mmol, New England Nuclear). Headspace subsamples were removed periodically for quantification of H_2, CH_4, $^{14}CH_4$, CO_2, and $^{14}CO_2$ as described below.

(ii) Pore water chemistry. Interstitial water was sampled either by squeezing water from subsections of cores (after ref. 17), or by equilibration of a dialysis membrane sampler (after ref. 9) filled with anoxic distilled water and incubated for 27 days in Bath Lake sediment. After removal of the dialysis membrane sampler, liquid samples were immediately removed with a helium-flushed syringe to helium-flushed butyl rubber-stoppered vials. Samples for sulfide analysis were transferred to helium-flushed vials containing 0.5 ml of 2.5% (wt/vol) cadmium acetate in 0.2% (vol/vol) acetic acid to preserve sulfide until analysis. Before methane analysis, dissolved gases were released from 3 ml of liquid samples by acidification with 0.5 ml of 50% H_2SO_4. Gaseous subsamples were removed with a gas-tight syringe (Hamilton) coupled to a mininert valve (Supelco) so that increased pressure did not result in loss of sample.

Analysis of headspace gases. Gaseous subsamples were removed from the headspace of tubes with a helium-flushed gas-tight syringe (Hamilton) and analyzed by gas chromatography-gas proportion analysis by a method similar to that described by Nelson and Zeikus (25). For analysis of H_2, CH_4, $^{14}CH_4$, CO_2, and $^{14}CO_2$ a Carle model 8500 thermal conductivity gas chromatograph equipped with a stainless steel column (3.2 mm outside diameter by 2.3 m [⅛ inch by 7.5 feet]) packed with 80/100-mesh Poropak N (Supelco) was coupled to a Packard model 894 gas proportion analyzer. Helium carrier gas flow through the gas chromatograph was 21 ml/min. Helium make-up gas was added after combustion (at 750°C) in the gas proportion analyzer to increase the total flow to 70 ml/min so that the flow of propane quench gas through the gas proportion analyzer was an optimal percentage (10%) of the total flow. The gas chromatograph was operated isothermally at 50°C. Peak height from gas chromatographic data and peak area from gas proportion analyzer data were compared to the responses of standards to determine gas concentrations and disintegrations per minute (based on standardization by liquid scintillation counting). The total amounts of H_2, CH_4, and $^{14}CH_4$ per tube were calculated by comparison of subvolume to the gas headspace volume. Total amounts of CO_2 and $^{14}CO_2$ per tube were determined by correction for the difference between subsample and headspace volume and also for gas solubility and dissociation equilibrium by the method of Stainton (32). Carbon dioxide rapidly released when samples were tubed under helium was subtracted so that only CO_2 evolved during incubation is presented. Because of variation in length of cores, and the restriction of activity to the upper surface layers, results are presented per core rather than on the basis of protein content or dry weight (see also reference 35).

More sensitive methane analysis was performed on a Varian 3700 series flame ionization gas chromatograph using a stainless steel column (3.2 mm outside diameter by 1.83 m [⅛ inch by 6 feet]) packed with 60/80-mesh Poropak Q (Supelco) with helium carrier flow at 40 ml/min and an isothermal oven temperature of 50°C. Injector and detector temperatures were 60 and 150°C, respectively.

Analysis of dissolved components. Sulfide (method 1.6 [33]) and sulfate (method 427C [2]) were determined on pore waters or on liquid samples removed after anaerobic incubations (sulfide preserved as cadmium sulfide before poisoning and analysis) by standard analytical procedures.

Volatile fatty acids were assayed by direct injection of liquid subsamples onto a Varian 3700 series gas chromatograph equipped with a glass column (0.2 mm inside diameter by 1.83 m [0.008 inch by 6 feet]) packed with 10% SP 1200/1% H_3PO_4 on 80/100-mesh Chromosorb W AW (Supelco). Isothermal oven temperature was 140°C, and injector and detector temperatures were 170 and 180°C, respectively. Helium carrier gas flow rate was 40 ml/min. Concentrations were calculated by comparison of peak area response to that of standards with a Spectra Physics model 4100 computing integrator.

Enumerations. Cores were subsectioned and tubed under helium in the field. Within a few hours, in the laboratory, subcores were suspended in prereduced liquid media prepared in roll tubes (11) designed to support growth of H_2-utilizing, methane-producing bacteria (see reference 35, except for the use of distilled water instead of Octopus Spring water and a final pH of 7.0) or sulfate-reducing bacteria (medium E [28]). Serial dilutions were prepared by transfer in helium-flushed syringes to establish a three-tube most probable number assay. After 53 days of incubation, methane-positive tubes were detected by gas chromatography as described above. After 30 days of incubation sulfate reduction was recognized by the formation of a black precipitate. Further incubation did not alter the results. Most probable number was determined with standard tables (2).

RESULTS

Response of whole cores to anaerobic incubation. In initial studies it was noted that substantial methane production did not occur until after anaerobic incubation of Bath Lake algal-bacterial mat whole cores for over 1 week (Fig. 1A). Carbon dioxide was rapidly produced after anaerobic incubation. Before the onset of significant methane production, an accumulation of hydrogen was noted. In the period before methanogenesis, the depletion of sulfate from an initially high level of 7.5 mM and hydrogen sulfide accumulation were suggestive of bacterial sulfate reduction (Fig. 1B). More sensitive methane analysis suggested methane accumulation during early incubation times (Fig. 2); however, the rate of methane accumulation was several orders of magnitude slower than after sulfate depletion.

During a similar incubation, accumulation of volatile fatty acids was noted after about 100 h of incubation (Fig. 1C). In particular acetate and propionate levels became increasingly detectable. Although other acid levels were low, there was also evidence that butyric, isobutyric, valeric, and isovaleric acids were present in higher concentration on extended incubation when compared to Formalin-killed controls. Formalin also prevented accumulation of gases in the headspace and sulfate reduction (see below).

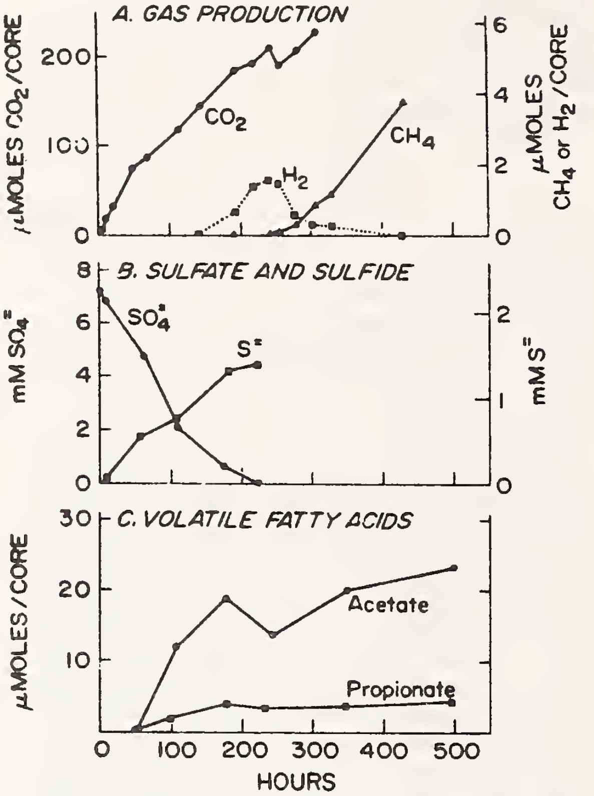

FIG. 1. *Chemical changes in response to anaerobic incubation of Bath Lake whole cores.*

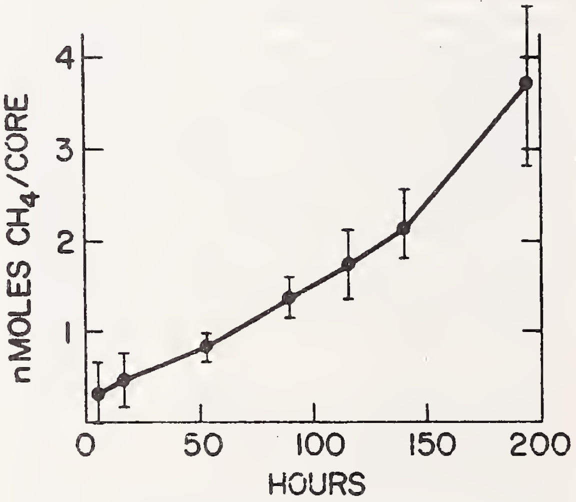

FIG. 2. *Methane accumulation after anaerobic incubation of Bath Lake whole cores. Bars indicating standard deviations from the mean are included because results were near the detection limits of the method.*

Fate of methane precursors in whole cores. When $NaH^{14}CO_3$ was added to samples tubed anaerobically, $^{14}CH_4$ was not detected until substantial methanogenesis had initiated (Fig. 3A). The addition of [2-^{14}C]acetate at varying times after anaerobic incubation (Fig. 3B) never resulted in generation of $^{14}CH_4$. When [2-^{14}C]acetate was added at the time of anaerobic tubing or after 140 h, rapid $^{14}CO_2$ production ensued. However, neither $^{14}CO_2$ nor $^{14}CH_4$ was produced when [2-^{14}C]acetate was added at about 260 or 430 h (after depletion of sulfate).

Anaerobic fate of methane. Extended anaerobic incubation of Bath Lake whole cores with $^{14}CH_4$ did not result in generation of $^{14}CO_2$. Even after 573 h, all added label was recovered as $^{14}CH_4$.

Depth distribution of anaerobic processes and bacteria. Sulfate reduction in subcores from different depth intervals was measured by following conversion of $Na_2{}^{35}SO_4$ to $H_2{}^{35}S$ during anaerobic incubation. Sulfate reduction was markedly higher in the surface interval (approximately 5 mm) than in all deeper layers (Fig. 4A). The most probable number of sulfate-reducing bacteria showed a similar depth distribution, with a difference of almost three orders of magnitude between the upper ($>10^6$ cells per subcore) and lower (10^3 to 10^4 cells per subcore) layers (Fig. 4B). Although 95% confidence limits are omitted for simplicity, a significant difference in population density is noted between the top and all deeper layers.

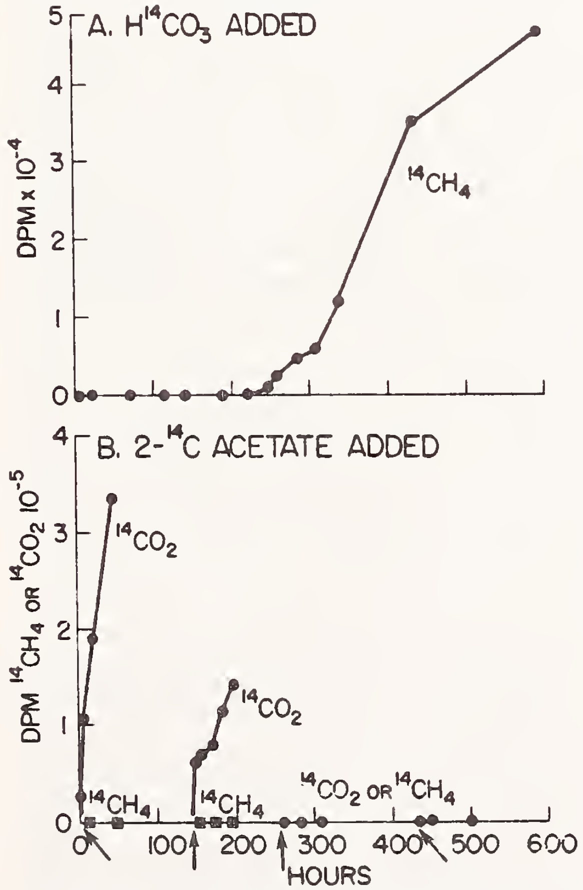

FIG. 3. *(A) Production of $^{14}CH_4$ (disintegrations per minute per core) from $NaH^{14}CO_3$ added immediately following anaerobic incubation of Bath Lake whole cores. (B) Production of $^{14}CO_2$ (●) or $^{14}CH_4$ (■) (disintegrations per minute per core) after addition of [2-^{14}C]acetate at various times (indicated by arrows) after anaerobic incubation of Bath Lake whole cores.*

Methane production in subcores from various depth intervals was below detection limits during the early incubation period. After sulfate depletion methane production increased in subcores taken from the 0 to 0.5-, 0.5- to 1.0-, and 1.0- to 1.5-cm depth intervals. In deeper layers no methane production was noted on extended incubation. Methane-producing bacteria were only detected in the upper 1.5-cm depth intervals, and the maximum population density was about 10^2 cells per subcore (0.5-cm interval).

A marked depth stratification was also noted for carbon dioxide production on anaerobic incubation (Fig. 5). Significant increases in carbon dioxide (after rapid initial outgassing of indigenous carbon dioxide) were only noted in the uppermost depth interval.

The predominance of sulfate reduction over methanogenesis was indicated in all depth intervals by the lack of rapid reduction of $NaH^{14}CO_3$ or [2-^{14}C]acetate to $^{14}CH_4$, and by the rapid oxidation of [2-^{14}C]acetate to $^{14}CO_2$ (Table 1).

Interstitial water chemistry. Sulfate, sulfide, and methane concentrations were determined after equilibration of a dialysis membrane sampler with sediment for 27 days. The results are presented in Fig. 6. A similar sulfate profile was observed on analysis of pore waters removed from subcores by squeezing. Sulfate levels in the water column were between 7.3 and 8.3 mM.

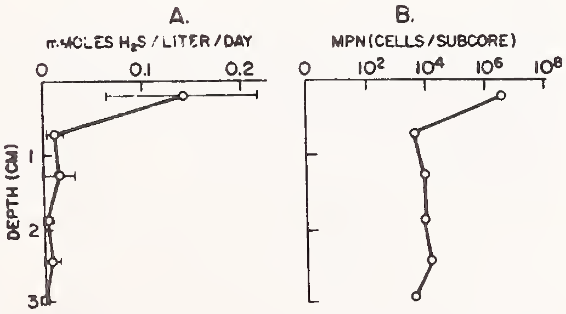

FIG. 4. *Sulfate reduction rate (A) and most probable number of sulfate-reducing bacteria (B) in various depth intervals of Bath Lake cores. Bars indicate standard deviation from the mean of analyses on four replicate core samples.*

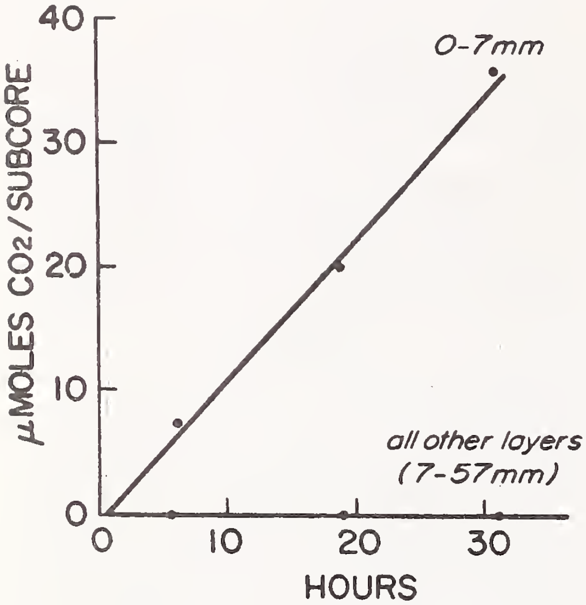

FIG. 5. *Carbon dioxide produced during anaerobic incubation of various depth intervals of Bath Lake cores. Indigenous carbon dioxide which rapidly evolved after tubing samples under helium was subtracted.*

TABLE 1. *Conversion of NaH$^{14}CO_3$ to $^{14}CH_4$ and of [2-^{14}C]acetate to $^{14}CO_2$ or $^{14}CH_4$ during a 6-h anaerobic incubation with various depth intervals of Bath Lake algal-bacterial mat cores*

Depth interval (mm)	$^{14}CH_4$ with NaH$^{14}CO_3$ added (dpm/subcore)	$^{14}CH_4$ with [2-^{14}C]acetate added (dpm/subcore)	$^{14}CO_2$ with [2-^{14}C]acetate added (dpm/subcore)
0-7	0	0	21,356
7-17	0	0	129,514
17-27	0	0	355,450
27-37	0	0	475,350
37-47	0	0	106,150
47-57	0	0	78,637

Although a sulfate minimum was observed in near surface layers, sulfate concentrations in deeper layers were similar to those of overlying water. A sulfide maximum was observed consistent with the sulfate minimum in near surface layers. Methane concentrations were higher within the algal-bacterial mat than in overlying water, but no consistent pattern was observed with depth in the algal-bacterial mat.

DISCUSSION

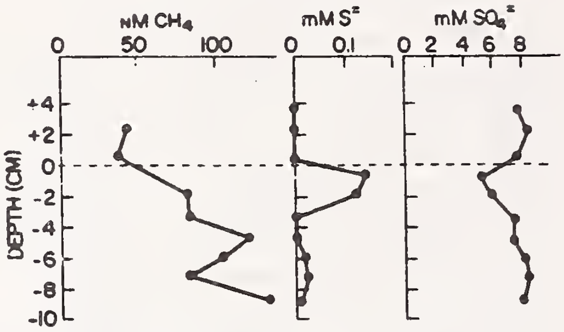

FIG. 6. *Depth profiles of methane, sulfide, and sulfate dissolved in pore waters of Bath Lake sediment. Dashed line indicates the interface between overlying water and the algal-bacterial mat. Pore water was sampled by 27-day equilibration of a dialysis membrane sampler with Bath Lake sediment.*

Viable populations of both sulfate-reducing and methane-producing bacteria were found to inhabit the Bath Lake algal-bacterial mat. Population densities of sulfate-reducing bacteria were from two to five orders of magnitude greater than those of the methanogenic bacteria. Very low initial rates of methanogenesis, lack of detectable $^{14}CH_4$ production from the methane precursors $H^{14}CO_3^-$ or [2-^{14}C]acetate until over 200 h of incubation, rapid oxidation of [2-^{14}C]-acetate to $^{14}CO_2$ (consistent with the activity of acetate-oxidizing, sulfate-reducing bacteria [36]), and rapid reduction of $^{35}SO_4^{-2}$ to $H_2{}^{35}S$ all support the conclusion that sulfate reduction is the dominant terminal anaerobic process in the decomposition of algal-bacterial organic matter in Bath Lake. The presence of viable methanogenic bacteria and slow but detectable increases in methane during early incubation indicate that the processes concur. Oremland and Taylor (27) and Winfrey and Ward (submitted for publication) reported concurrent methanogenesis and sulfate reduction in nearshore subtidal and intertidal marine sediments where sulfate is also high. However, a respiratory index for [2-^{14}C]-acetate ($^{14}CO_2$/($^{14}CO_2$ + $^{14}CH_4$) [38]) of 1.0 indicated the dominance of sulfate reduction over methanogenesis in the Bath Lake system. The data of Fig. 2 and 4 were used to estimate initial rates of 0.24 nmol of CH_4 produced per core per day and 0.14 mmol of H_2S produced per liter per day (280 µmol of H_2S per core per day) (assuming a 2-ml liquid volume in sulfate reduction rate experiments). Assuming that eight electrons are required in sulfate reduction and methane production (valid only if all methane came from reduction of CO_2), electron flow through either process can be calculated by multiplying the reaction rate times eight electrons per mole of product. Comparison of rates of electron flow showed that sulfate reduction was more than 1,000 times greater than methane production in the initial incubation period. Other anaerobic respirations such as denitrification were not studied, but may be involved in anaerobic decomposition in this environment. Comparative studies in another high-sulfate system (marine sediments) indicated that nitrate reduction as well as sulfate reduction was involved in anaerobic oxidation of organic matter (30).

The marked increase in methanogenesis upon sulfate depletion suggests that methane production is limited by the activity of sulfate-reducing bacteria. It is important to emphasize that because interstitial sulfate levels were always high, increased methane production after sulfate depletion is an artificial condition which is likely never to occur in the natural environment. The increase in methanogenesis occurred despite high levels of H_2S (>1.25 mM), further suggesting that the basis for lower initial rates of methanogenesis in Bath Lake sediment was not inhibition by H_2S as suggested by Cappenberg (3, 4). The production of $^{14}CH_4$ from $H^{14}CO_3^-$ but not from [2-^{14}C]acetate and the accumulation of acetate suggest that acetate may not be an important energy source for methanogenic bacteria in Bath Lake sediment. It is also interesting to note that acetate accumulation preceded sulfate depletion indicating a breakdown between acetate-producing and acetate-consuming reactions. This observation and the rapid uptake of [2-^{14}C]acetate into cellular material in Bath Lake (unpublished data of this laboratory) suggest that acetate consumption by terminal members of the anaerobic food chain may not be the dominant mode of acetate utilization in this environment. The accumulation of acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate indicates that these compounds may be fermentation intermediates during anaerobic degradation; however, accumulation of the more reduced acids may be a result of disrupted electron flow by decreased removal of H_2 (20) or acetate (Kemmerling and Ward, manuscript in preparation).

The low levels of methane in sulfate-rich marine sediment zones have been attributed to possible anaerobic oxidation of methane (29). In this system $^{14}CH_4$ was not converted to $^{14}CO_2$ even on incubation over 500 h. The low rates of methane production suggest that low concentrations of dissolved methane in Bath Lake sediment are more likely due to lack of production than to anaerobic methane oxidation.

The depth stratification of the terminal anaerobic process sulfate reduction, sulfate-reducing bacteria, and carbon dioxide production was striking. Similar results were found for the depth distribution of sulfate reduction (7, 8, 10, 13–15, 24, 31, 34; Winfrey and Ward, submitted for publication) and sulfate-reducing bacteria (8, 15, 24, 31) in marine (or other high-sulfate) sediments and in cyanobacterial mats of a hypersaline lake (16). Interestingly, the restricted activity of methanogenesis to near surface layers in low sulfate sediments (see 20) and in a low-sulfate hot spring algal-bacterial mat (35) was also noted. Methanogenic activity after sulfate depletion and methane-producing bacteria were also maximal in near surface sediment in Bath Lake. The rapid conversion of [2-^{14}C]acetate to $^{14}CO_2$ and not to $^{14}CH_4$, and the lack of rapid reduction of $H^{14}CO_3^-$ to $^{14}CH_4$ in all depth intervals were further evidence of the inactivity of methanogenic bacteria in deeper layers where sulfate reduction was less active. The exclusion of methane production to deeper layers where sulfate is depleted has been suggested based on interstitial water chemistry in marine sediments (20). Although sulfate is not depleted by sulfate reduction in Bath Lake sediments, the maximal activity of methanogenesis in near surface layers suggests that both terminal anaerobic processes are limited to upper sediment layers. Similar results were reported by Winfrey and Ward (submitted for publication) for intertidal marine sediments. The basis for this stratification may be the availability of organic matter which becomes decomposed as it is buried to deeper sediment layers.

Pore water chemistry indicated a near surface sulfate minimum but not sulfate depletion. As in marine sediments, sulfate depletion would be expected if ongoing sulfate reduction decreased sulfate pools. The lack of sulfate depletion may be due to replenishment of sulfate by diffusion from overlying water because the depth interval studied was not large. Another possible mechanism for replenishment of sulfate may be the reoxidation of H_2S in anoxygenic photosynthesis. Castenholz (6) reported that sulfide-adapted *Spirulina labyrinthiformis* from Bath Lake could use sulfide to support photosynthesis when the herbicide DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] inhibited photosystem II. The ability of another phototroph of the mat, *Chloroflexus aurantiacus*, to use sulfide as an electron donor in photosynthesis has also been reported (19). In this regard maximal sulfate reduction, the sulfate minimum, the sulfide maximum and phototrophic organisms all occur in the uppermost layer of the mat.

To the extent that comparisons can be made, anaerobic degradation of algal-bacterial organic matter in Bath Lake appears to resemble organic matter decomposition in marine sediments. Carbon and electron flow is dominated by sulfate-reducing bacteria whose activity limits methanogenesis to an insignificant role in terminal anaerobic processing of organic matter. Both processes and populations are markedly reduced at depth, possibly due to lack of available organic matter. Differences from marine sediments in carbon and electron flow (i.e., acetate incorporation) and chemistry (i.e., lack of sulfate depletion with depth) may be related to the proximity of phototrophic microorganisms with the capability for photoheterotrophic growth and photosynthetic H_2S oxidation.

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Final Report for Project 435-811

covering the period 6/29/81 - 6/29/82

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I. FINAL TECHNICAL REPORT

INTRODUCTION

The general objective of our research in this and previous grant periods has been to understand the role of microorganisms in controlling the stability and efficiency of digesters used to convert animal wastes to methane. The practical outcome of our work could potentially be 1) better knowledge of how to design and operate digesters for maximum efficiency of conversion of waste to methane, and 2) improved methods to recognize and control the impending failure of digesters. In earlier grant periods we have focused on dairy cow manure digestion as a model system. We have sought to understand the microbiological explanation for the observation that failing, or inefficient, digesters accumulate propionic acid (see Figure 1). The accumulation of propionic acid is presumably related to an imbalance between microorganisms involved in its production and consumption. This imbalance could be related to a decrease in the number of a critical bacterial population that cannot reproduce fast enough to avoid being washed out of a rapid throughput (short retention time (RT)) digester. It could also be caused by an imbalance in the flow of metabolites among the collection of bacteria which carry out complete decomposition to methane. Such an imbalance could be due to more rapid production than consumption of a metabolite when one microbial group is more active than another (e.g., after over feeding of a readily fermentable material). In previous grant periods our approach did not permit us to conclude which cause explained inefficiency and digester failure. For example, in the experiment in Figure 1, the RT was short (3.3 days) but the digester was also stressed by overfeeding, so that the reason for propionate accumulation could have been due to either cause. In the present grant period we studied these two possibilities separately.

During this period we completed studies on the effect of short retention times on digester stability. If a microorganism is unable to reproduce as fast as fluid flows through the system, it will eventually be washed out. We isolated the effect of short retention time on population washout by reducing the retention time of a digester using an appropriately reduced feed concentration so that the feed loading remained constant. Since the total amount of feed was constant, the only stress to the system was the reduced RT. In previous grant periods, digester instability was maintained by decreasing the retention time with the feed concentration held constant. This created two stresses on the system: 1) decreased RT and 2) increased feed loading. It was of interest to isolate these stresses to determine if one is more important in digester failure than the other.

We also investigated how an imbalance in material flow among microorganisms involved in decomposition might cause propionate to accumulate. Some background on the microbiology of anaerobic decomposition is provided in Figure 2 which summarizes the processes of balanced and unbalanced digesters and emphasizes the interrelationships among the various groups of microorganisms (based on Bryant, 1976, 1979). Hydrolysis of polymers (eg. cellulose) to monomers (sugars) is followed by fermentation. These reactions result in the production of volatile fatty acids (VFA's) (eg. acetate, propionate), hydrogen, and carbon dioxide. Methane is produced by methanogenic bacteria mainly from the splitting of acetate to methane and carbon dioxide or the reduction of carbon dioxide with hydrogen. Acetate is the only VFA that is used by the methanogens (Mah, et al., 1977). Propionate, butyrate, and the other VFA's are degraded by a group of bacteria termed "obligate hydrogen-producing acetogenic bacteria" because both hydrogen and acetate are products of VFA catabolism (Boone and Bryant, 1981; McInerney, et al., 1981). Under standard conditions, the acetogenic reactions are thermodynamically unfavorable. It has been postulated that to make them thermodynamically favorable the removal of breakdown products (acetate, hydrogen) is necessary. Removal of H_2 also influences fermenting microorganisms to produce more acetate and less propionate (or other reduced acids) (Wolin, 1974; Miller and Wolin, 1979). Recent results support this model of anaerobic decomposition (Lovely and Klug, 1982; Mackie and Bryant, 1981), and the sensitivity of propionate accumulation to increases in acetate and H_2 concentration (Kaspar and Wuhrmann, 1978).

Methanogens, then, play a key role in the maintenance of stable digesters by consuming the products of fermentation and

VFA degradation (acetate, hydrogen). As long as these products are at low enough concentrations acetogenesis from the higher VFA's is thermodynamically favorable and fermentation leads mainly to production of acetate and not propionate. The two question marks in Figure 2 (bottom) indicate the points where acetate and/or H_2 could influence propionate accumulation. Studies in previous grant periods focused on the effects of acetate on propionate degradation. In this grant period we focused on the effects of dissolved hydrogen and acetate on propionate production (by fermentation) and consumption (by acetogenesis) since the accumulation of these compounds could affect either fermentation or acetogenesis. We investigated two approaches to creating unbalanced conditions in the model digester. Using H_2 accumulation as an indication of unbalanced conditions we tested overfeeding of fermentable materials (e.g., cellulose, starch) and inhibition of methanogenesis as ways to stress material flow in the digester. Inhibition of methanogenesis led to more reproducible H_2 accumulation and was used to test the basis for propionate accumulation. We used 2-bromoethanesulfonic acid (BES), a very specific inhibitor of methanogenesis (because it is a chemical analog of Coenzyme M which has been found only in methanogens), to inhibit methanogenesis and test the effect of H_2 and acetate accumulation on fermentation and acetogenesis. Propionate acetogenesis was determined by adding radioactive propionate and analyzing the resulting gases and liquids for radioactivity.

We have modified our methods for determining propionate turnover by replacing 1-^{14}C-propionate (used in previous grant periods) with 3-^{14}C-propionate because questions have been raised about the pathway of propionate degradation. Using 3-^{14}C-propionate we could follow the loss of propionate and the appearance of ^{14}C-acetate, $^{14}CH_4$, and $^{14}CO_2$ and determine propionate turnover regardless of pathway. We also performed studies using ^{14}C-cellulose to determine the flow of carbon from cellulose to the products of anaerobic waste digestion (VFA's, CH_4, CO_2). The effect of H_2 on the fermentation of cellulose was determined by observing the ratios of volatile fatty acids produced in the presence compared to the absence of BES.

METHODS

Maintenence of digesters

Methods for maintaining the dairy cow manure digesters were given in previous completion reports with the exception of the reduced retention time--constant loading experiment. In this experiment, two one liter digesters were initiated with manure gathered from a pen of nonmilking heifers that were being fed only alfalfa hay (see Table 1). This manure was chosen because the low concentrations of VFA's and low concentrations of easily fermentable substrates (i.e., grain) should minimize the stress from organic loading. The two digesters were started from pooled effluents of older dairy cow manure digesters (see previous reports) at a 10 day RT by removing 100 ml (effluent) and feeding 100 ml of freshly thawed manure (influent). The digesters were monitored for VFA levels, gas production rate, gas composition (% methane), and pH. When stability was reached, as indicated by no further changes in the monitored parameters, a retention time reduction experiment was initiated with one digester by removing 200 ml effluent and returning 100 ml thawed manure plus 100 ml tap water. This procedure reduced the retention time to 5 days but kept the feed loading rate the same [ie. the digester continued to receive 100 ml of manure daily (see Table 2)]. The other digester was maintained at a 10 day RT to serve as a control. Subsequent RT reductions to 4, 3, and 2 days were performed on the digester after stability was reached at the previous RT. Since the % solids (and thus buffering capacity) decreased with decreasing RT we found it necessary to add sodium bicarbonate to maintain pH near neutrality at RT's shorter than 10 days (see Table 2).

Radiolabelling experiments

Effluent from a 10 day RT dairy cow manure digester (8.8% solids) was taken just before feeding and transferred to the reaction vessel described below. Liquid samples (3 ml) were removed at various times before or after addition of BES and sealed anaerobically in two dram vials under prereduced 50%He/50%CO_2 according to Hungate (1969). Radioisotopes (0.1ml 10uCi/ml 3-^{14}C-propionate, 6.3 mCi/mmole, or UL-^{14}C-cellulose, 7.7 uCi/mg, New England Nuclear, sodium salts) were added from prereduced sterile stock solutions to begin an experiment. After 2hr incubation at 35°C reactions were terminated by addition of 0.1 ml formalin. Methods for analysis of radiolabel in gases and VFA's are described below.

Gas Analysis

Gas production in digesters was monitored by collecting the gas produced daily in gas-impermeable bags and measuring the gas volume as described by Varel, et al. (1977).

Dissolved H_2 was initially measured directly in liquid samples of a digester fluid using a method described by Robinson, et al. (1981). A different method for measuring headspace hydrogen concentrations was developed and used for calculating dissolved hydrogen concentrations. This method required a stirred chamber to ensure rapid equilibration of gases between the liquid and gas phases. It consisted of a plexiglas reaction chamber (Figure 3) that has a water jacket for maintaining constant temperature and the blades from a blendor (Waring, VWR Scientific) for constant stirring. The top was sealed with a rubber stopper with three holes bored in it for 1) venting, 2) removing gas samples for analysis, and 3) removing liquid samples. Gas samples were taken with a Glaspak syringe (Becton Dickenson) coupled to a Mininert teflon valve (Supelco) and stripped of CO_2 by the method of Robinson, et al. (1981). CH_4 and ^{14}C-gases were analyzed by gas chromatography (GC)--gas proportional counting (GPC) and corrected to a per tube basis as described by Ward and Olson (1980). H_2 was determined using a 2.3 m x 1/8" OD stainless steel column packard with 80/100 mesh Poropak N (Supelco) in a Carle Model 8500 thermal conductivity GC. N_2 was the carrier gas (21 ml/min) and the oven temperature was 50°C.

Analysis of Liquid Components

VFA's and pH were measured as described in previous reports. A method for concentrating ^{14}C-labelled VFA's was developed for use in radiolabelling experiments. The liquid in the vials from radiolabelling experiments was centrifuged for 10 minutes at 10000 RPM (Sorvall RC2-B). The supernatant was filtered through a 0.45um Millipore filter. 1.0 ml of the filtrate was transferred to a 1.5 ml microcentrifuge tube. 50 ul of 2N NaOH was added to each tube to raise the pH above 10, preventing the volatile loss of the VFA's and 20 ul of 50 mM hexanoic acid was added as an internal standard. The samples were completely dried overnight at 60°C. 200 ul of 35% meta phosphoric acid (HPO_3) was added to dissolve the VFA's and acidify to pH 4 . Tubes were vortexed well to assure complete recovery of VFA's. The drying followed by reconstitution in a smaller volume effectively concentrated the VFA's by a factor of 5. The concentrated liquid was injected into a Varian 3700 GC interfaced with a Packard model 894 GPC. Operating characteristics of the GC were as follows: injector-170°C, detector-250°C, oven initially 105°C for 2 min, programmed to increase at 40°C/min to 145°C for the final 3 min. Output of the Varian GC was integrated by a Spectra Physics 4100 computing integrator. Output of the Packard GPC was integrated by a Spectra Physics Minigrator. ^{14}C-VFA's were corrected for concentration and then to a per tube basis.

RESULTS AND DISCUSSION

Retention Time Reduction Experiments

The results of the reduced retention time experiment are shown in Figures 4 and 5. Digester A was the experimental digester and digester B served as the control digester because it's RT was one step behind digester A (ie, when digester A was at 5,4,3,and 2 day RT, digester B was at 10,5,4,and 3 days RT respectively). Digester B also provided an opportunity to repeat the observations made on digester A. Decreasing the RT caused a change in all of the parameters that were monitored. The gas production rate decreased by 50% when RT was shifted from 10 days to 5 days. Continued shifts caused further decreases in gas production proportionate to the decrease in % solids at shorter RT (see Table 2). The decrease in gas production may have occurred because of the reduced time that solids remain in a digester at a shorter RT. There was a corresponding decrease in the efficiency of conversion of waste fed to all products of fermentation (Table 3). It is interesting that in earlier experiments (see previous completion reports) decreasing RT (when loading was not held constant) did not lead to decreased gas production rate or fermentation efficiency. The difference may relate to the composition of manures in these two experiments. In the experiments of Figures 4 and 5, manure was from nonmilking heifers fed entirely a hay diet. In the previous experiment manure was from milking cows fed a high percentage grain diet. The milking cow manure would thus contain high levels of starch which is much more readily fermentable than the lignin-bound cellulose likely to be a major fermentable polymer of the heifer waste. Due to an accident in feeding the digesters in this experiment we had an opportunity to witness the importance of manure composition on gas production. The dashed lines of Figures 4 and 5 indicate a time period when the digesters responded to accidental feeding of milking cow manure. Note that gas production rapidly increased in digester A (at 4 day RT) and digester B (at 5 day RT). The conversion efficiency also increased during this period (Table 3). The conversion efficiencies reported in Table 3 are very low in comparison to those reported earlier for milking cow manure (ca. 33%, see previous reports). This may be another indication of the nonstressing nature of heifer waste which should contain less readily fermentable organic polymers. The gas composition also changed from 55% methane at 10 day RT down to 27% at 2 day RT. This may have resulted from the addition of bicarbonate as a pH buffer which would have increased the total CO_2 in the digester at shorter RT and thus the % CO_2 in equilibrium with dissolved CO_2 species.

Since the main purpose of this experiment was to determine if a short RT would cause a washout of a critical microbial population (i.e., propionate-degrading acetogenic bacteria) and an accumulation of propionate, we were looking for an increase in propionate relative to acetate. Such a change in VFA's occurred rapidly, for example, in the experiment reported previously (Figure 1), where it could not be ascertained whether the imbalance was due to increased loading or reduced RT. Although VFA's increased in concentration with decreasing retention time, acetate increased relative to propionate. Thus, it appears that the accumulation of propionate is not a direct result of a short retention time. We apparently did not wash out a critical microbial population responsible for maintaining digester stability (low propionate/acetate ratio). Again, it was interesting to see the effect of accidental feeding of milking cow manure on the VFA levels at constant, but short RT's (dashed lines of Figures 4 and 5). In both digesters A (4 day RT) and B (5 day RT) VFA's had stabilized and acetate was much higher than propionate before the accidental feeding. Immediately after the accidental feeding VFA's increased, but propionate increased relative to acetate. 10-20 days after giving the incorrect feed acetate and propionate levels were essentially equivalent. This is further evidence that the stress to the digester had more to do with the imbalance in material flow among microorganisms than with population washout. After returning to heifer manure, acetate returned to levels much higher than propionate.

Experiments on Production and Consumption of Propionate

H_2 measurement. An alternate explanation for propionate accumulation is that increased levels of H_2 and/or acetate could have a negative effect on propionate acetogenesis and/or could increase the production of propionate from fermentation. From our work in previous grant periods it seemed that increased feed loading caused saturation of degradative reactions causing the substrates of those reactions (H_2 and acetate) to accumulate (see Figure 2, bottom). We attempted to measure dissolved H_2 directly in the liquid phase using the method of Robinson, et al.(1981). The results showed that H_2 increased with time after daily feeding of a digester (Figure 6). However, we were concerned about the method because H_2 levels seemed too high and variable. We were able to show that H_2 followed the distribution predicted by Henry's Law of gas solubility in distilled water (Figure 7), and in sterile manure (Table 4) in a well stirred reaction chamber (see Methods). Using the reaction chamber we showed that measurements of dissolved H_2 in biologically active manure by gas stripping of liquid samples (after Robinson, et al. (1981) was impossible as levels detected were orders of magnitude above levels predicted by Henry's Law (Table 4). This was not a function of the method of poisoning the sample as heat killing, acidification and formalin all led to detection of H_2 12, 44, and 88 times respectively that predicted by Henry's Law. The levels of H_2 measured in the liquid were compared to levels of H_2 measured in the headspace in a separate experiment. The results in Figure 8 demonstrate the accumulation of H_2 following addition of cellulose to stress a digester. The problem with measuring the H_2 in the liquid by the method of Robinson, et al. (1981) becomes obvious when it is considered that the dissolved H_2 level calculated is $< 2\%$ of the headspace levels reported in Figure 8. We chose to use the headspace measurements of H_2 to calculate dissolved H_2 concentrations in all further experiments.

Because we had difficulty in reproducibly forcing H_2 accumulation by addition of various polymeric compounds (e.g., starch, celluose) to stress a digester, we began experiments on the effect of a methanogenesis inhibitor (BES) on H_2 accumulation. Figure 9 shows that BES caused CH_4 production to stop and H_2 to accumulate. Figure 10 shows the kinetics of H_2 accumulation in the headspace of the reaction chamber following BES addition. This gave us a reproducible system in which to investigate the effect of accumulating H_2 and acetate on reactions involved in propionate formation and degradation.

Effect of BES on cellulose fermentation and propionate acetogenesis. A method was developed to follow propionate acetogenesis without regard to the pathway by which it occurred, because prior assumptions about the pathway may not have been valid (see previous reports). Figure 11 shows that propionate turnover in manure could be followed by observing the loss of 3-^{14}C-propionate and the formation of ^{14}C-acetate, $^{14}CO_2$ and $^{14}CH_4$. Figure 12 shows the the effect of BES on propionate turnover. Before BES addition, 3-^{14}C-propionate was completely degraded because none was detected after a 2 hour incubation. ^{14}C-acetate, $^{14}CO_2$ and $^{14}CH_4$ were detected in high amounts. After BES was added, ^{14}C-propionate was detected at significant levels and lower levels of $^{14}CO_2$ and $^{14}CH_4$ were detected. There did not appear to be a significant difference in this result if the propionate turnover experiment was run 2, 4 or 7 hours after BES addition. This suggests that although H_2 continued to increase (see Figure 10), the environment for propionate degradation did not become progressively worse. It is interesting that BES did not completely stop propionate turnover. It is possible that the H_2 concentration was not high enough even at 7 hours after BES addition to cause complete inhibition of propionate degradation. However, calculations on the thermodynamics of propionate degradation (see Thauer, et al. [1977]) using the data from the ^{14}C-propionate and ^{14}C-cellulose experiments (see below) suggested that the propionate degradation reaction was beyond the thermodynamic limit ($\Delta G>0$) under our experimental conditions.

Figure 13 is a graph of the effect of BES on ^{14}C-cellulose degradation. High H_2 concentrations should cause an increase in propionate production if H_2 has the expected effect on fermentation (see Introduction). Before BES addition, ^{14}C-cellulose was converted to ^{14}C-acetate, ^{14}C-propionate, $^{14}CO_2$ and $^{14}CH_4$. The addition of BES decreased the production of $^{14}CO_2$ and $^{14}CH_4$. Since inhibition of methanogenesis caused accumulation of acetate, it is not surprising that a corresponding increase in ^{14}C-acetate was observed. BES did not cause an increase in ^{14}C-propionate or the ratio ^{14}C-propionate/^{14}C-acetate, suggesting that high H_2 levels may not have had an effect on propionate production in cellulose fermentation.

The results of these experiments may suggest that propionate acetogenesis is more sensitive to BES addition, and the corresponding accumulation of H_2 and acetate, than is fermentation. We consider these results preliminary, however, and need to repeat these observations under conditions in which we know that propionate has begun to accumulate. If this finding is correct, it is interesting that H_2 accumulation preceeds the cessation of propionate degradation by several hours. H_2 increase may thus be a better monitor of impending digester failure than propionate accumulation, because it is the earliest sign of imbalance among the microorganisms involved in the overall waste conversion to methane. It will be important to see if there is a minimum H_2 and/or acetate level required to stop propionate degradation completely.

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Figure 1. Long term changes in volatile fatty acids in a 3.3 day retention digester.

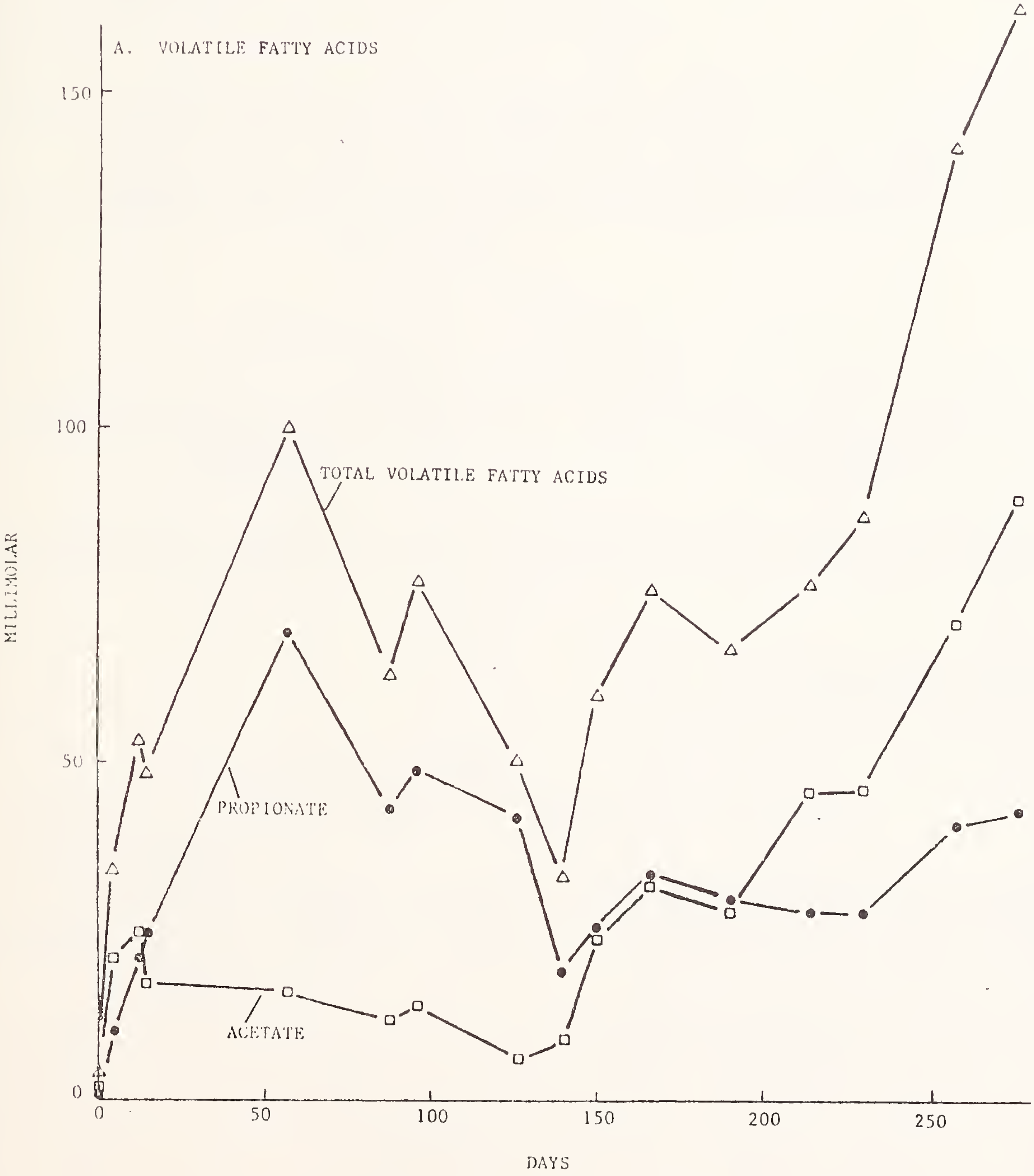
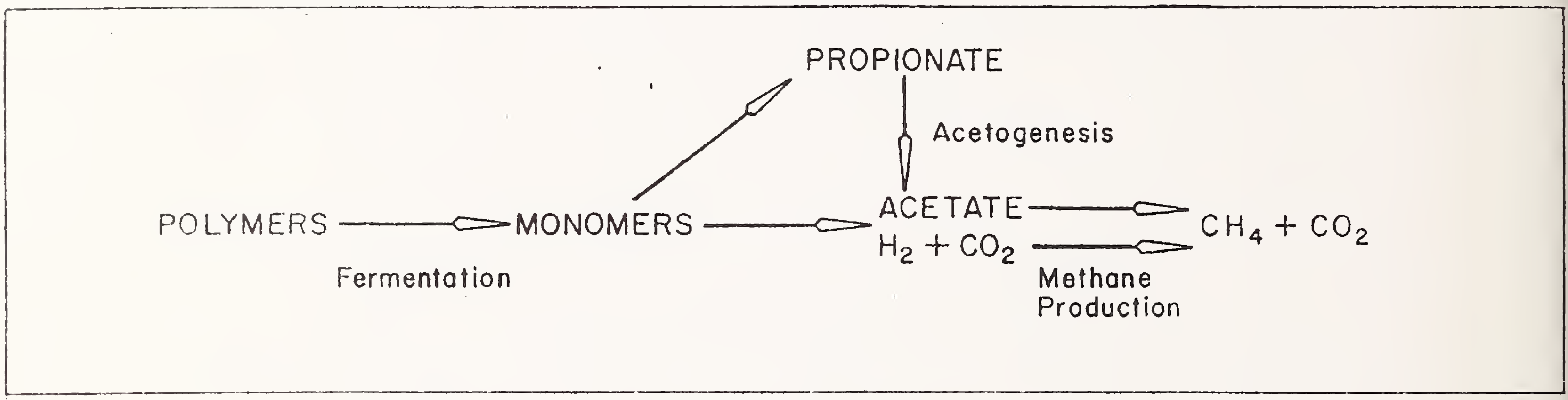


Figure 2. Models for balanced anad unbalanced operation of digesters for dairy cow manure conversion to methane.

BALANCED OPERATION



UNBALANCED OPERATION

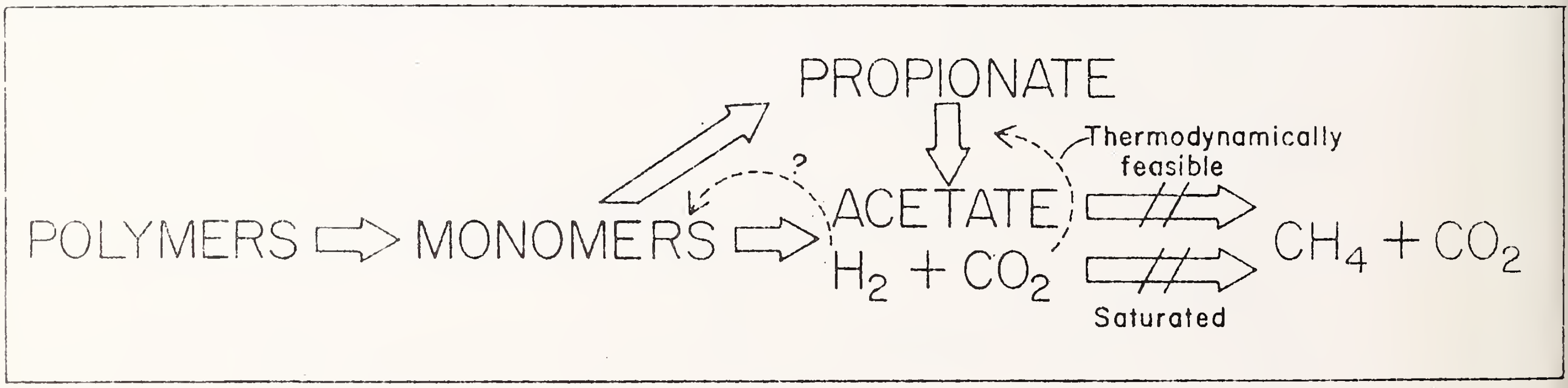


Figure 3. Schematic of plexiglas reaction chamber

a. Blending assembly.

b. Water jacket - inlet and outlet connected to circulating water bath to maintain temperature.

c. Serum bottle stopper - septum for removing gas headspace samples.

d. Silicon tubing (1 mm I.D.) - for removing liquid samples.

e. Vent for maintaining atmospheric pressure inside chamber. Vented into high ionic strength low pH water (20% NaCl, 0.5% citric acid) to prevent entrance of O_2, and to prevent gases from dissolving in the gas trap.

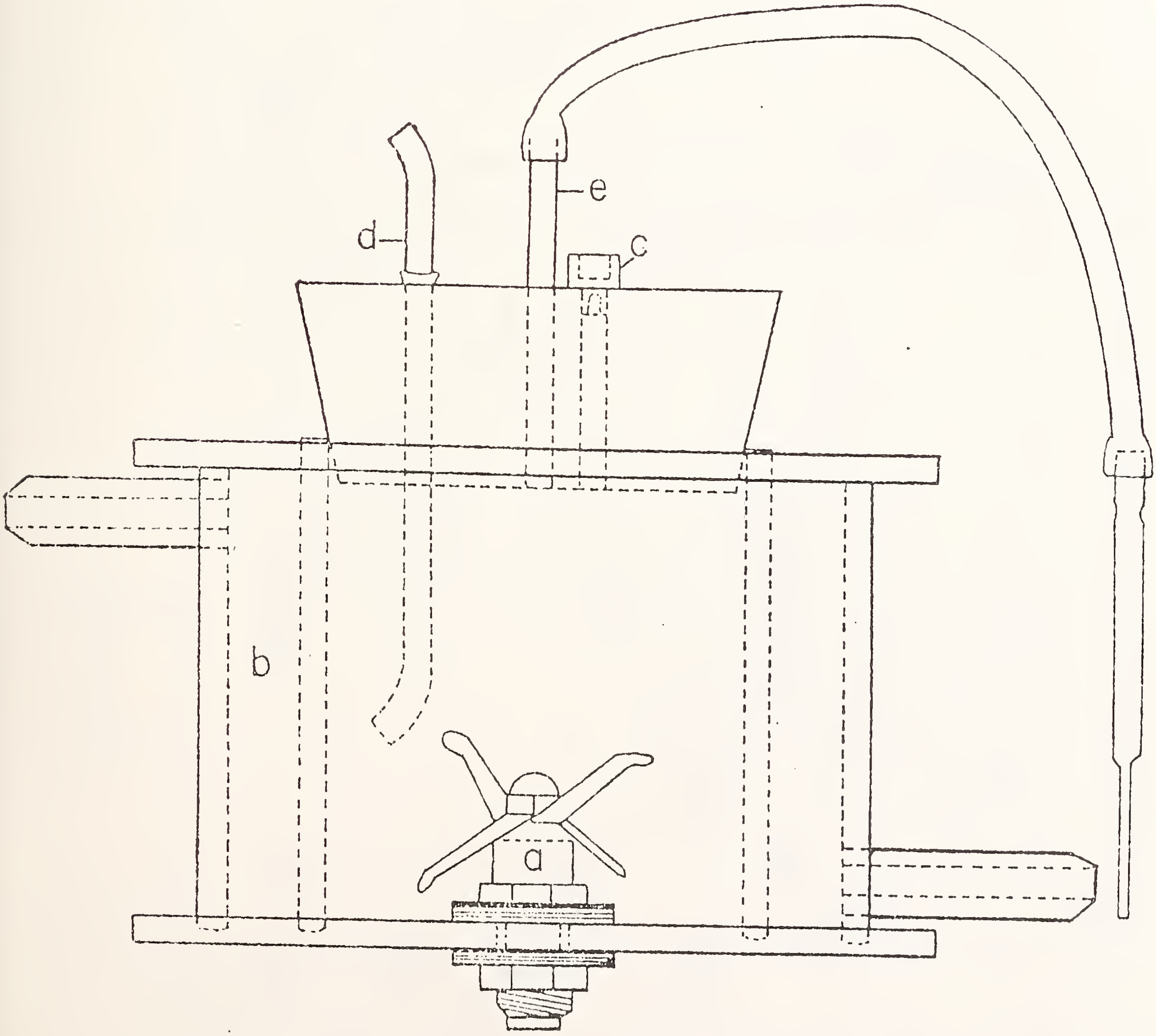


Figure 4. Digester A gas production rate and VFA concentrations from retention time reduction experiment. Numbered areas between vertical dashed lines indicate retention times. Dashed lines connecting data points indicate the effects of accidental feeding of milking cow manure.

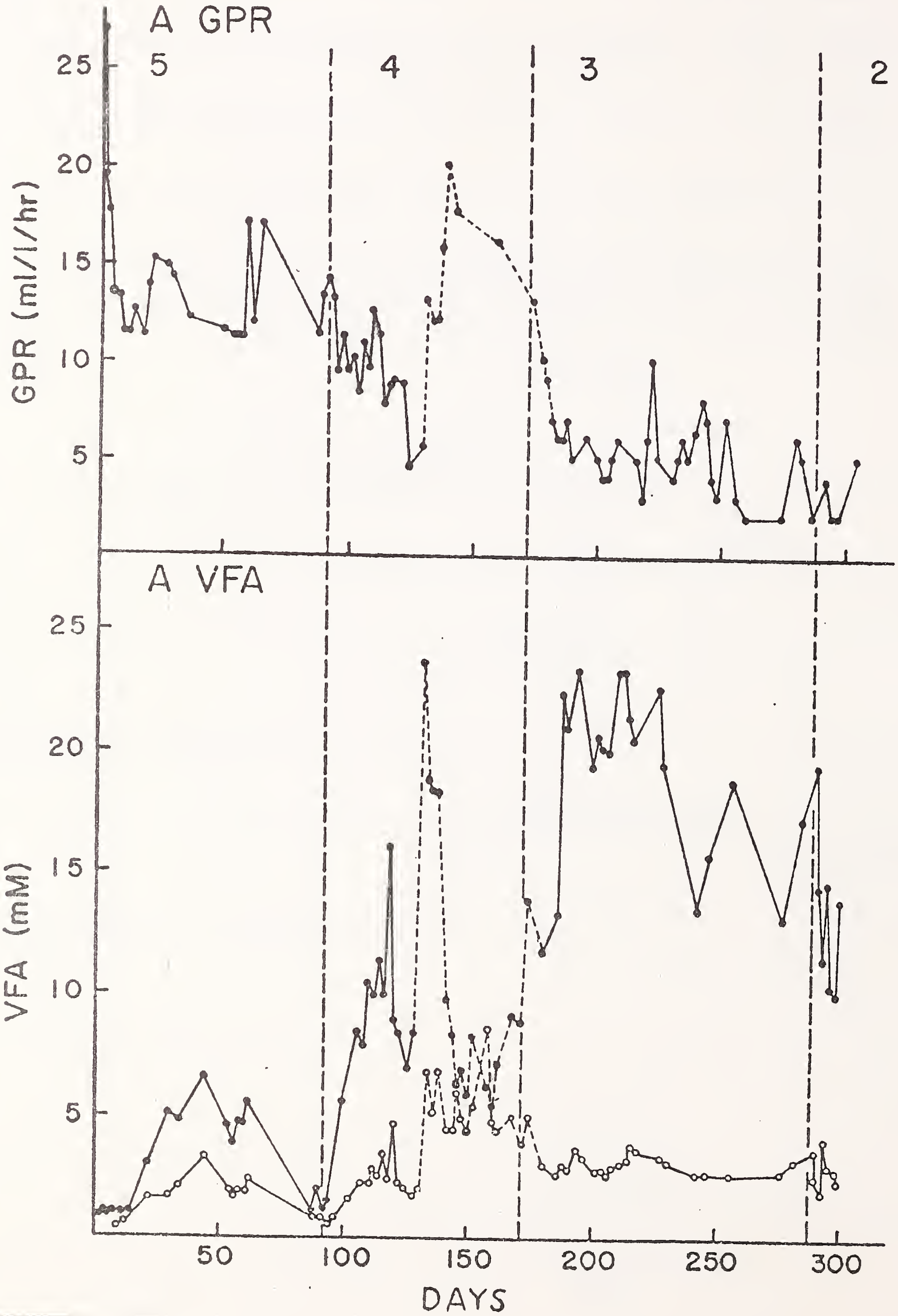


Figure 5. Digester B gas production rate and VFA concentrations from retention time reduction experiment. Numbered areas between vertical dashed lines indicate retention times. Dashed lines connecting data points indicate the effects of accidental feeding of milking cow manure.

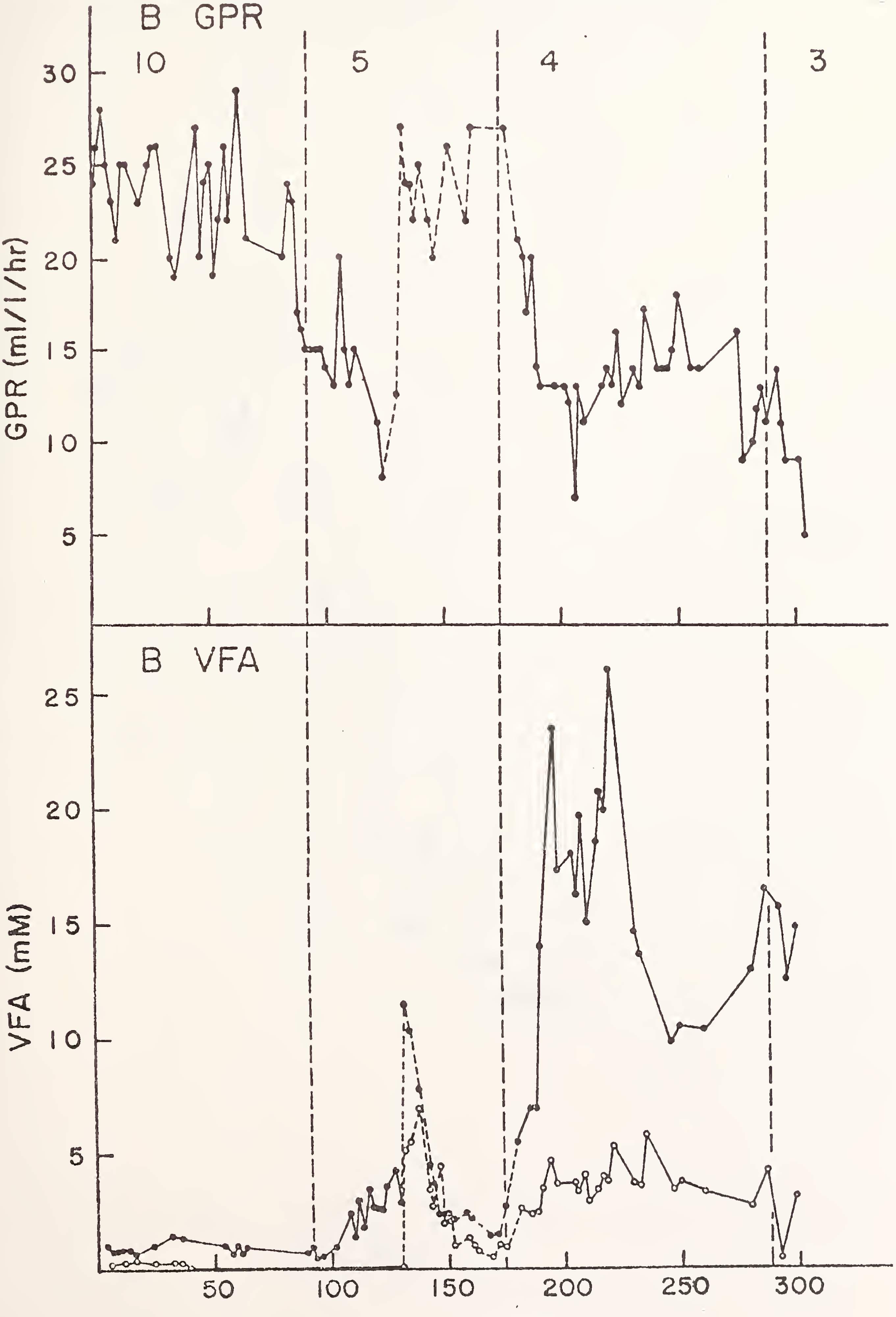


Figure 6. Changes in the mean H_2 concentration with time after feeding in a digester fed 8.8% dairy cow manure at a 5 day retention time. Stars indicate observations which were significantly greater ($p \leq .05$) than H_2 concentration at one hour before daily feeding.

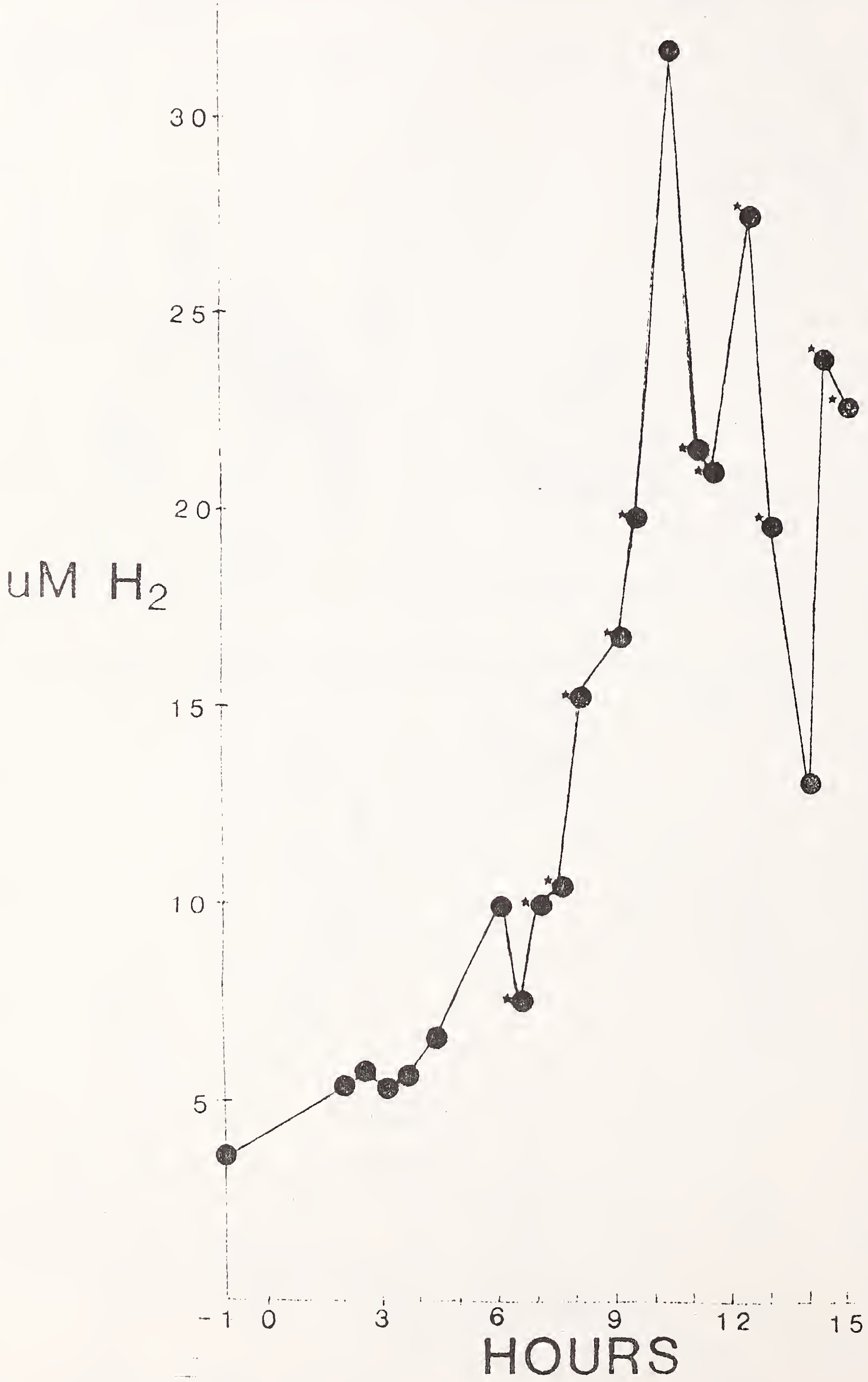


Figure 7. Concentration of H_2 dissolved in distilled water in equilibrium with various concentrations of H_2 in the gas headspace. Pearson correlation coefficient for the line drawn is 0.988. Bars represent one standard deviation.

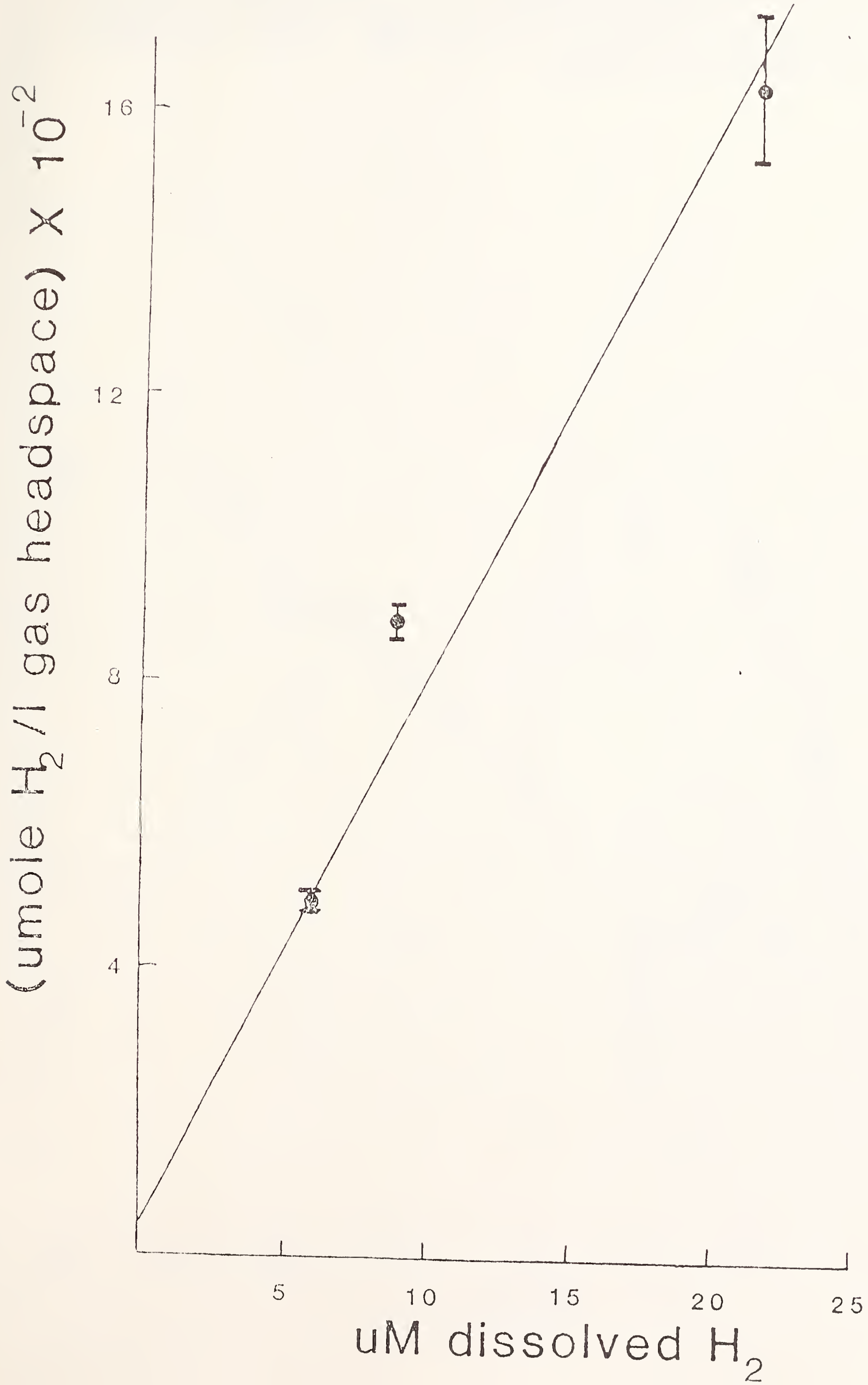


Figure 8. Changes in dissolved and headspace H_2 concentration with time following feeding of an experimental dairy cow manure (10 day RT, 8.8% solids, milking cow manure) digester with 1.67% (w/v) cellulose. Bars represent one standard deviation

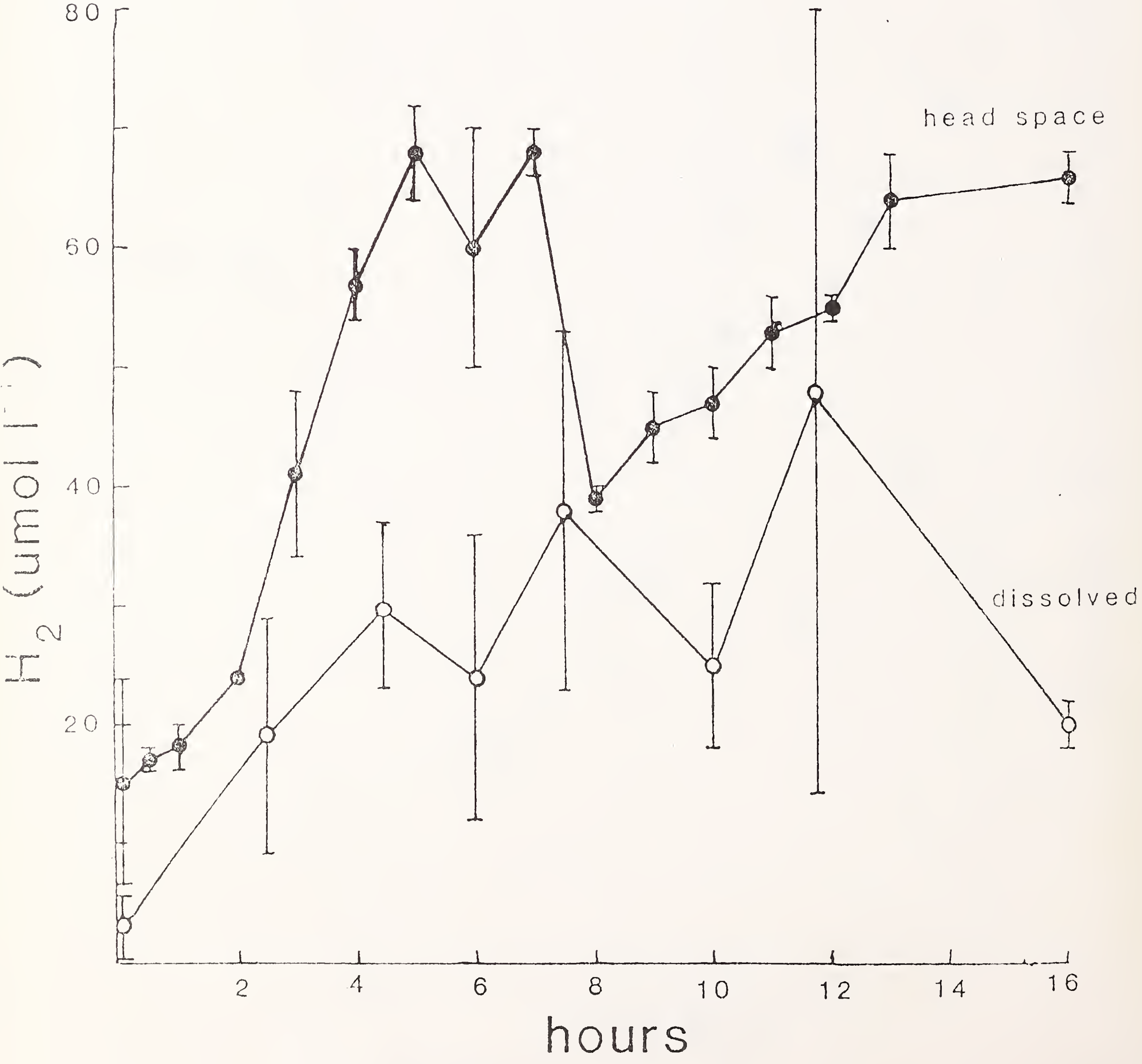


Figure 9. The effect of bromoethanesulfonic acid (50 mM) on H_2 and CH_4 concentrations per ml of gas he space from a 10 day RT digester 4 hrs after feeding 8.8% manure. Bars when present represen one standard error; when absent the error is smaller than the symbol.

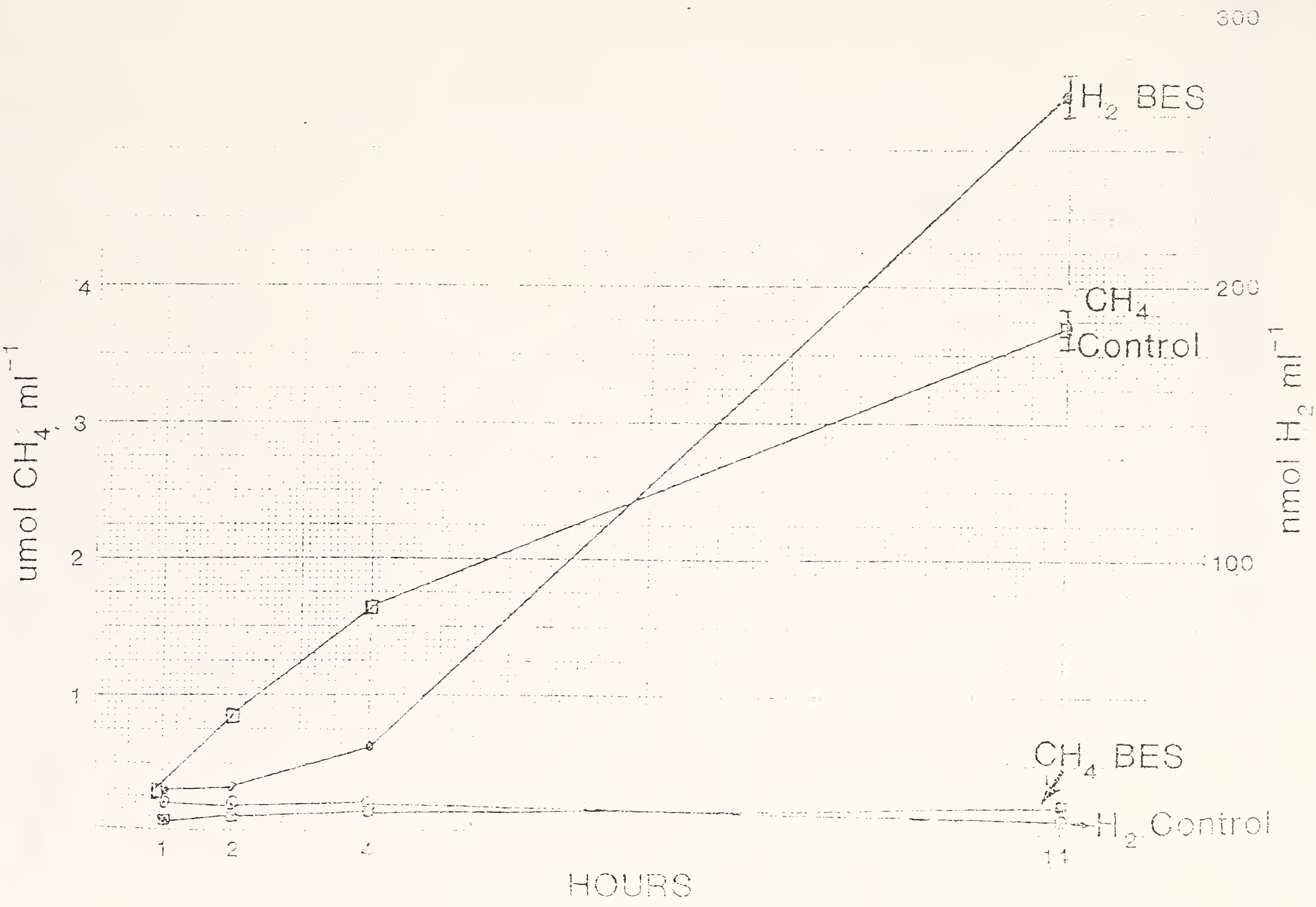


Figure 10. The effect of Bromoethanesulfonic acid (0.1 M) on H_2 in the headspace of the reaction chamber. The manure used was from a 10 day RT digester fed 8.8% milking cow manure.

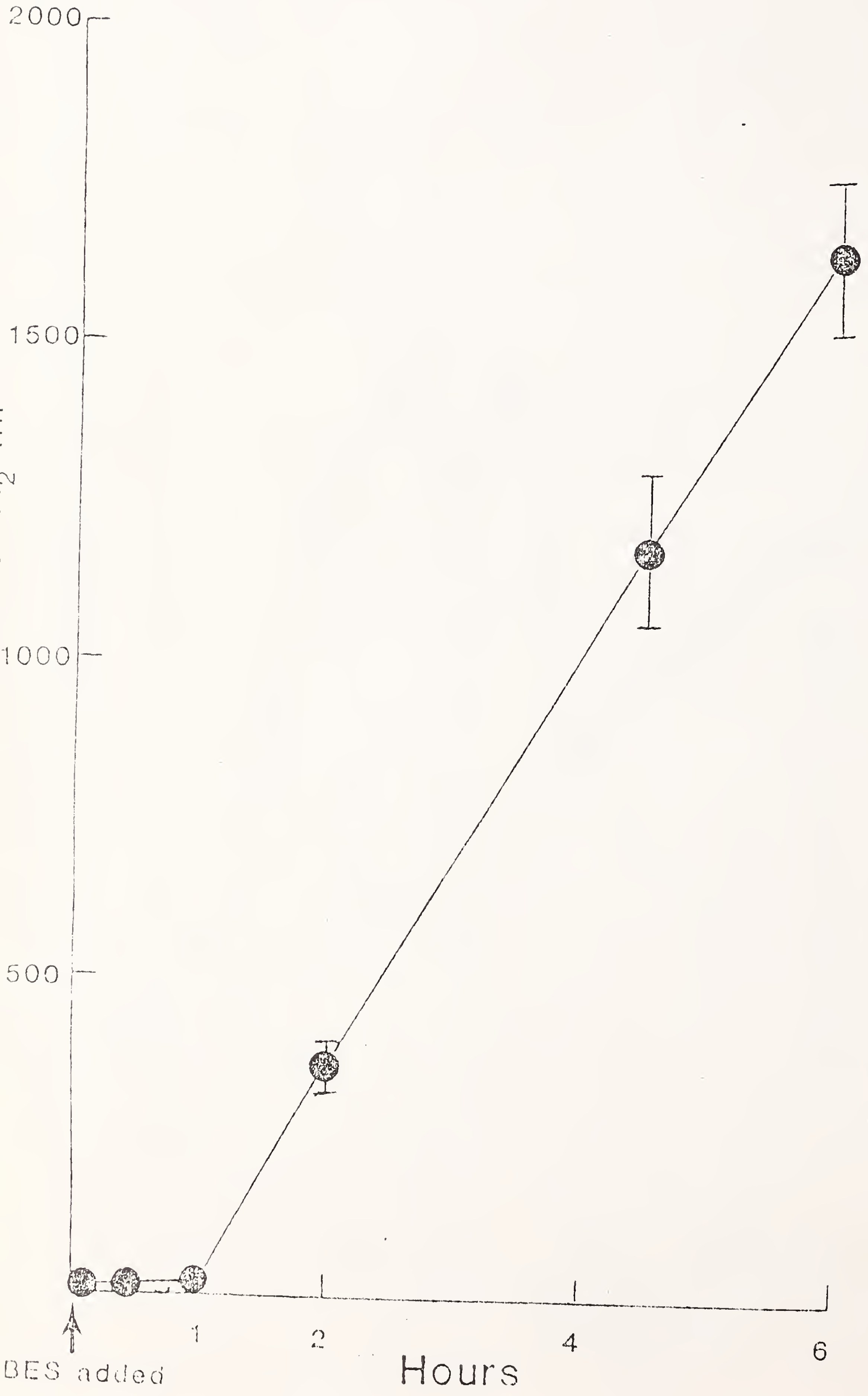


Figure 11. Time course for the turnover of 3-^{14}C-propionate to ^{14}C-volatile fatty acids and ^{14}C-gaseous metabolites. Bars indicate ± one standard error.

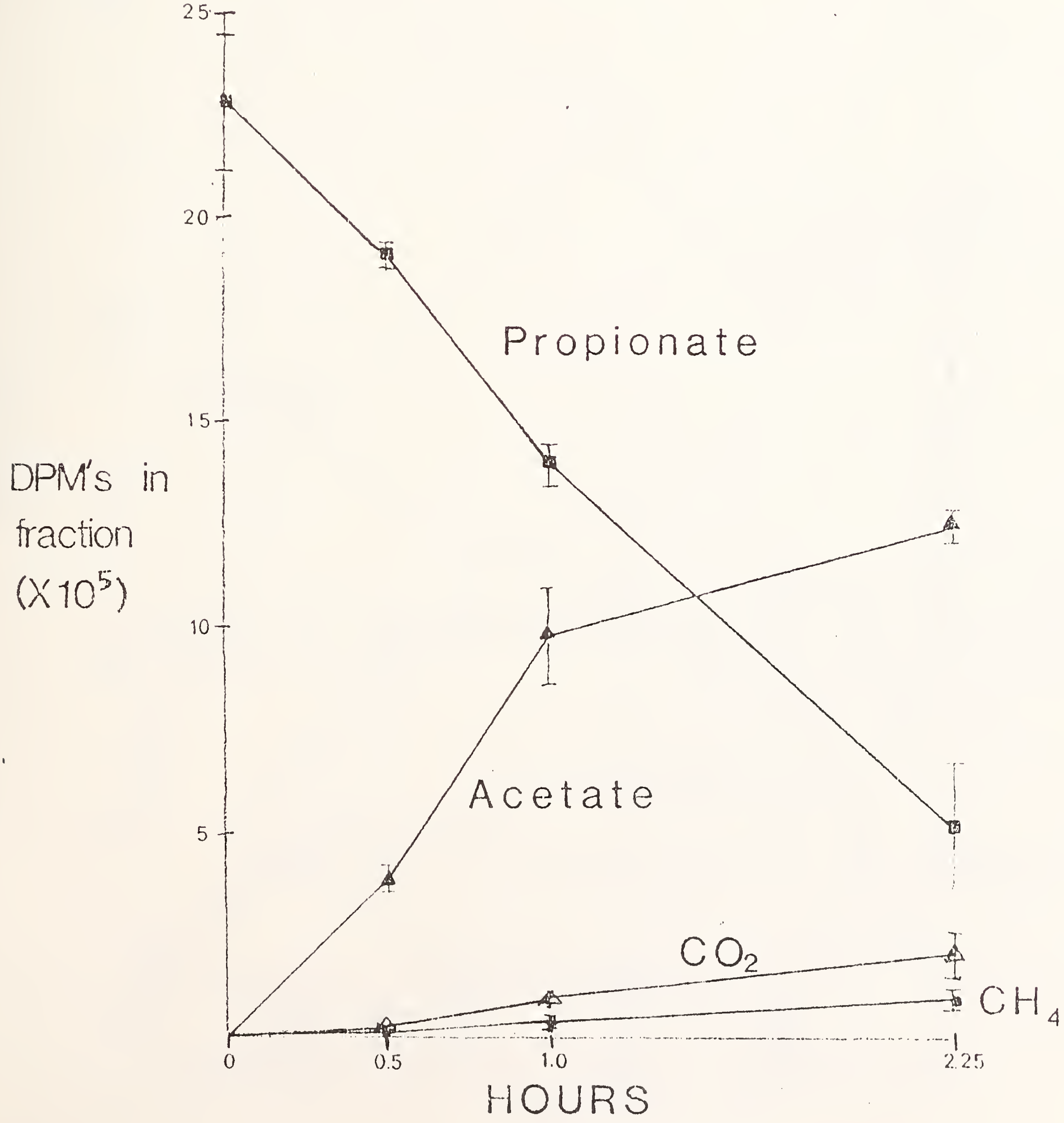


Figure 12. Effect of 2-bromoethanesulfonic acid (50 mM) on fate of 3-^{14}C-propionate.

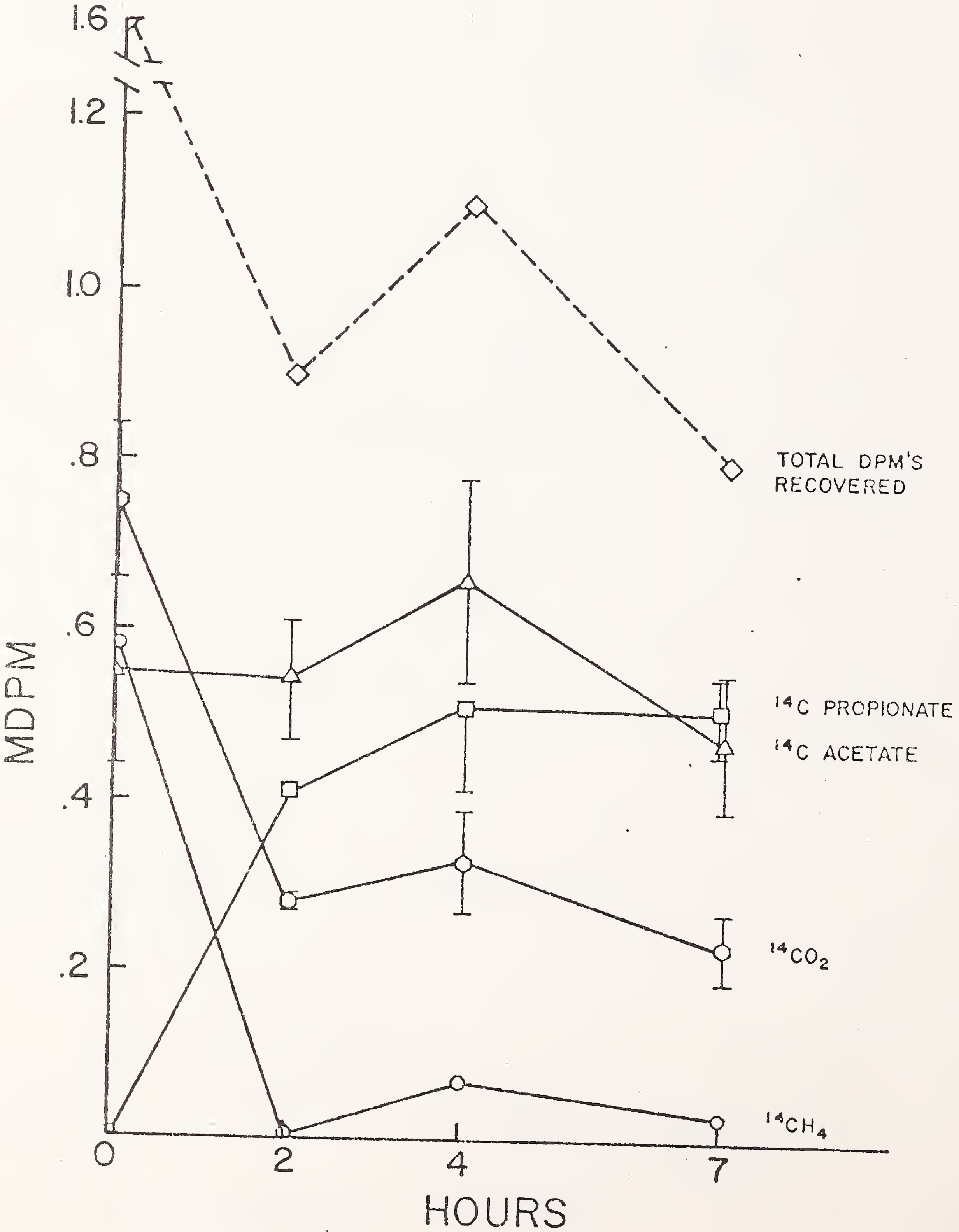


Figure 13. Effect of 2-bromoethanesulfonic acid (50 mM) on fate of UL-^{14}C-cellulose.

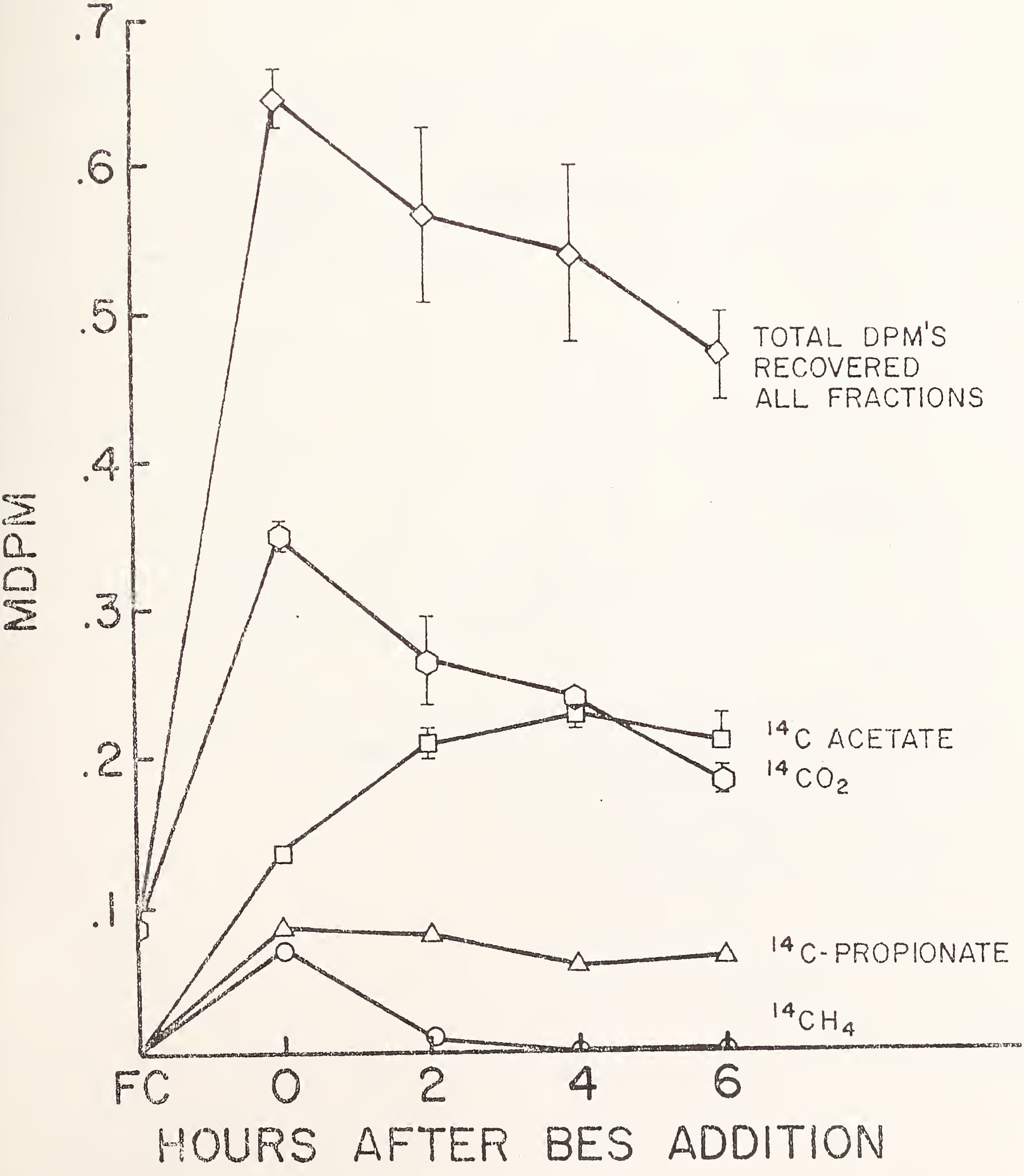


Table 1. Composition of Manures.

Characteristic	Non-milking Heifer Manure[a]	Milking Herd Manure[b]
VFA's (mM)		
Acetate	17.50	47.42
Propionate	3.00	5.88
i-Butyrate	0.44	0.28
n-Butyrate	0.79	2.73
i-Valerate	0.37	0.23
n-Valerate	0.32	0.53
pH	7.2	7.2
% solids	8.8	8.8

[a] Used only in the retention time reduction experiments.

[b] Used in all other digesters.

Table 2. Method for preparing manure for the retention time reduction experiments. Note the amount of manure fed remains constant but the final % solids and RT changes.

Retention Time (days)	ml 8.8% Manure Added	ml H_2O[a]	Final % solids
10	100	0	8.8%
5	100	100	4.4%
4	100	150	3.52%
3	100	233	2.64%
2	100	400	1.76%

[a] When pH dropped below 7.0, 1% w/v $NaHCO_3$ was added to the water to serve as a buffer.

Table 3. Efficiency of conversion of waste to fermentation products at various retention times in the retention time reduction experiment.

Retention Time (days)	% Efficiency[a] Digester A	 Digester B
I. Nonmilking heifer manure		
10	----	8.00
5	5.00	6.67
4	4.05	5.08
3	2.91	3.20
2	3.01	----
II. Milking cow manure[b]		
5	----	10.22
4	8.49	----

[a] % efficiency =

$$100 \times \frac{\text{gC in gases recovered/day} + \text{gC in VFA recovered/day}}{0.4 * \text{gC in volatile solids fed/day}}$$

[b] Efficiencies calculated with data from those days (see figures 4 and 5) when milking cow manure was accidentally fed to the digesters normally receiving nonmilking heifer manure.

reviewed the results of previous grants.

3) Publicity. We have discussed our results with scientific colleagues who visited this year (Dr. David Boone, Alberta Research Council, and Dr. James Tiedje, Department of Crop and Soil Science, Michigan State University), and at the national meeting of the American Society for Microbiology. Several individuals and companies interested in our work made inquiries during the grant period. We responded by sending copies of published work. One paper was published this year as a result of work previously funded by this program, and a copy is appended. We collaborated with A.E. Montana, Inc. of Amsterdam on several occasions when our expertise and equipment could be used to assist in operational problems at the plant.

4) Expenditures. Attached to this report is a request to the MSU Grants and Contracts office to submit Budget Detail Forms as requested, if this has not already been done. MSU has invested several thousand dollars in graduate student stipend to B. Rutherford to maintain the continuity of the project before and after this grant period.

5) Labor.

a. One full time graduate student has contributed approximately 2500 hours, 2000 of which were funded by this grant.

b. Three work study students contributed approximately 460 hours in total funded by this grant.

c. The principal investigator contributed approximately 10% of his time (200 hrs) but grant funds were not expended for this purpose. The total number of person hours for the grant period was approximately 3160 hrs (2460 funded by this grant).

6) Recommendations On the basis of our results we offer the following suggestions to those operating manure digesters. We caution, however, that these suggestions are preliminary and further testing is required as mentioned above.

a. The chemical composition of manure is variable. Since unbalanced conditions may be caused by overfeeding, it is necessary to optimize a digester in terms of feed concentration (%solids) and retention time (RT) for each waste. A solids percentage and RT which is not stressing for one waste may be inappropriate for a waste which has readily fermentable material such as starch. At a given RT, solids concentration may be increased to achieve the maximum rate of waste conversion to methane, so long as volatile fatty acid levels (especially propionate) remain low (<5mM).

b. When a digester fails it accumulates propionic acid. pH is the least desirable parameter to monitor to control against failure because it's decrease occurs as the digester fails. An increase in propionate relative to acetate is a better index of impending failure, but requires analysis of volatile fatty acids. H_2 may increase before propionate and thus may be the earliest index of failure. Dissolved H_2 should be calculated from H_2 measured in the headspace of a stirred digester and not directly

Table 4. Hydrogen measurements in sterile liquids and manure[a]

Condition	ml added H_2[b]	H_2 (Headspace)[c]	H_2 (Liquid)[d]	Experimental[e] K_H		(X 10^{-4})
distilled water	1	124.33	9.649	8.15		
	2	227.76	3.351	6.30	$\bar{X} = 6.8$	1.15
	3	336.28	18.919	6.03		
sterile[e] manure	1	135.10	14.004	11.10		
	2	236.367	12.681	5.75	$\bar{X} = 7.74$	2.92
	3	327.72	19.480	6.38		
biologically active manure						
heat killed		3.80	33.24	.0935		
acid killed		2.07	35.12	.181		
formalin kill		1.43	42.87	.63		

a. Manure was from a 10 day RT digester and was strained through two layers of cheesecloth to remove the larger solids.

b. H_2 had to be added to the distilled water and sterile manure because none was being produced; the biologically active manure produced H_2 and no extra H_2 was added.

c. nmol/0.2 ml of gas headspace; mean of triplicates

d. nmol/ml of liquid; mean of triplicates

e.
$$K_H = \frac{H_2 \text{ in liquid (nmol/ml)} \times 760 \text{ mm Hg}}{(H_2 \text{ in headspace (nmol/.2ml)} \times 5) \times 22.4 \text{ l/mol} \times 646 \text{ mm Hg}}$$

Theoretical Henry's Law constant is $K_H = 8.12 \times 10^{-4} - 7.81 \times 10^{-4}$

II. ADDITIONAL INFORMATION REQUESTED FOR FINAL REPORT

1) Actual progress of project. The first section of this document is a detailed report of technical results obtained during our research project. It can be seen that the work completed followed the schedule outlined in the original grant proposal. Studies on the effects of reduced retention times were completed within the grant period. Methods for H_2 analysis and detection of ^{14}C-labelled volatile fatty acids were developed and later applied to begin to investigate the basis for propionate accumulation in an artificially unbalanced digester. Our results leave us with a preliminary view of the problem of digester stability as it relates to retention time and the accumulation of propionate. Because of the potential applications of our findings (see below), we need to confirm the observations made in the basis of present data. This will be done by Bill Rutherford during the completion of his Master's thesis. We will provide MDNRC with a copy of the thesis when it is available later this year. Specifically, we need to test the hypotheses that 1) digesters can be run at very low retention times on a long term basis, 2) H_2 accumulation preceeds propionate accumulation and may be the best indicator of impending digester failure, and 3) the basis for propionate accumulation is the same when a digester is imbalanced by overfeeding instead of artificial inhibition of methanogenesis.

2) Involvement with the MDNRC The only contact was a visit by Georgia Brensdal just after the grant period began. We mainly

in the liquid.

c. Retention times as short an 2-3 days do not seem to be too fast for digester operation so long as the feed concentration is not too great. As mentioned above, the composition of the waste may influence the %solids and RT required for stability. For alfalfa hay fed heifer waste at 1-2% solids, 2-3 day RT's were stable for short time periods. Further testing is ongoing to determine if such short RT digesters have long term stability.

7) Other information requested in Appendix E of general grant provisions.

Item 1 was addressed in items 4 and 5 above. Items 2 and 3 do not apply to this research grant.

Item 4. B. Rutherford has taken a part time position at A.E. Montana, Inc. as a result of his collaboration with them in the past year. He expects to work there full time when his thesis is completed.

Item 5. We have avoided the usual spinoff projects which normally occur during the course of a research project, for the sake of completing this project. As mentioned above (item 1), we expect to complete our continued effort on this problem in the next few months and intend to inform MDNRC of our final results. B. Rutherford has developed an interest in increasing the cost effectiveness of biofuel production by utilization of otherwise wasted resources during conventional fermentation reactions. I would not be surprised to see him work on this at A.E. Montana, Inc. as I know they are already interested in this possibility.

Item 6. The following ideas were previously expressed during Georgia Brensdal's visit (see above). I believe that funding basic research on applied problems is a worthwhile investment of MDNRC funds. This should be considered an investment in improving energy alternatives from the present state of the art. If this type of investment is continued, MDNRC should consider some problems of research as opposed to developmental projects. Short grant periods are impractical in a detailed scientific investigation, and continuity is essential because of the need to provide continuous support for students, and to maintain equipment. I have been funded for three one-year grant periods on this project. The project itself has been going on since 1977. On several occasions we have had to absorb the cost of the project on other funds because of administrative delays and changing personnel. Research proposals should be funded over longer periods to account for the normal development of a research project. An effective system might be longer term funding with release of funds for subsequent years dependent on demonstration of research productivity. This is the system used by the National Science Foundation. It also ensures that a program will not have to endure abrupt losses of funds, unless it is not a productive program.

I would also encourage MDNRC to request outside reviewers for research proposals, since the proposals are often too technical to be effectively evaluated in house. Reviewer(s) could be solicited from the authors of literature cited in a research proposal. This would help ensure that the ideas expressed in such a proposal are considered interesting to scientific peers, as well as to Montanans. Since the result of an investment in research is usually less applicable in the immediate sense than a demonstration project, it would be more difficult to establish the potential effectiveness of a research proposal without external review.

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Temperature Adaptations in the Terminal Processes of Anaerobic Decomposition of Yellowstone National Park and Icelandic Hot Spring Microbial Mats

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The optimum temperatures for methanogenesis in microbial mats of four neutral to alkaline, low-sulfate hot springs in Yellowstone National Park were between 50 and 60°C, which was 13 to 23°C lower than the upper temperature for mat development. Significant methanogenesis at 65°C was only observed in one of the springs. Methane production in samples collected at a 51 or 62°C site in Octopus Spring was increased by incubation at higher temperatures and was maximal at 70°C. Strains of *Methanobacterium thermoautotrophicum* were isolated from 50, 55, 60, and 65°C sites in Octopus Spring at the temperatures of the collection sites. The optimum temperature for growth and methanogenesis of each isolate was 65°C. Similar results were found for the potential rate of sulfate reduction in an Icelandic hot spring microbial mat in which sulfate reduction dominated methane production as a terminal process in anaerobic decomposition. The potential rate of sulfate reduction along the thermal gradient of the mat was greatest at 50°C, but incubation at 60°C of the samples obtained at 50°C increased the rate. Adaptation to different mat temperatures, common among various microorganisms and processes in the mats, did not appear to occur in the processes and microorganisms which terminate the anaerobic food chain. Other factors must explain why the maximal rates of these processes are restricted to moderate temperatures of the mat ecosystem.

Interest in the thermophilic conversion of organic wastes to methane has increased in recent years, as thermophilic digestion of organic matter is more rapid and seems to be more efficient than mesophilic digestion (12, 21, 25). Studies on decomposition in natural high-temperature environments are potentially useful in understanding thermophilic bioconversion processes (32). The algal-bacterial mats present in the effluent channels of neutral to alkaline hot springs provide such an environment. A thick (1 to 4 cm) mat develops between the upper temperature limit for photosynthesis (ca. 73°C in Yellowstone National Park and ca. 60°C in Iceland) and the upper temperature limit for metazoan grazers of the mat (40°C) (6, 10, 29). Doemel and Brock (13) suggested that Yellowstone National Park algal-bacterial mats undergo nearly complete decomposition. The isolation of *Methanobacterium thermoautotrophicum* from the Octopus Spring mat (31) and the observation of active methanogenesis in this mat (26) suggested that a complete anaerobic food chain is present. Previous reports indicated that maximum decomposition (13) and methanogenesis (26) occurred between 48 and 59°C. Studies of the Octopus Spring mat at 65°C and *M. thermoautotrophicum* isolated from the same site suggested that methanogenic bacteria are optimally adapted to the temperatures at which they are found (33). Many other microorganisms and processes which occur in this environment are optimally adapted to the temperatures at which they are found along the thermal gradient (2, 8, 20).

This study was undertaken to determine whether decomposition of hot spring microbial mats, as indicated by the microbial activities which terminate the anaerobic decomposition process, is, in general, greatest at the moderate temperatures of the mats. Several springs were surveyed, including one Icelandic spring in which sulfate reduction dominated methane production as a terminal process in anaerobic decomposition. Adaptation to environmental temperature was also investigated as a possible explanation for the observation that decomposition is greatest at temperatures well below the upper temperatures for mat formation.

MATERIALS AND METHODS

Study areas. Three alkaline (pH 8 to 8.6) siliceous hot springs in the Lower Geyser Basin of Yellowstone

National Park were investigated. The locations of Octopus, Twin Butte Vista, and Mushroom Springs were given by Brock (6). A fourth study area was an artificial channel constructed by Fraleigh and Wiegert (14) in the Serendipity Springs group, in which the effluent of the spring (pH 6 to 7) is diverted so that it flows at a constant rate down a plywood channel (1.2 m wide by 24 m long). Sulfate concentrations ranged from 0.12 to 0.22 mM in these springs.

A microbial mat of one Icelandic hot spring was also investigated. The spring was located in the Hveragerdi region above the west bank of the river Grendalsa, about 1 km upstream of its confluence with the river Varma. The effluent of the spring flows along the gentle incline of a bench above the river Grendalsa and enters the effluent channel of a major group of acid springs located just to the north. It is probably the spring designated 168B by Castenholz (11). The mat in this spring grew at <60°C. The source temperature was 97 to 98°C. The source water had a sulfate concentration of 0.88 mM and a pH of 6 to 7.

Sampling and incubation. Whole cores were removed from the mat with a no. 4 brass cork borer (50.3 mm^2) and transferred directly to 1-dram (1.77-gm) glass vials, which were sealed anaerobically under helium (method described in reference 15, with the modification described in reference 26). Anaerobically sealed samples from hot springs in Yellowstone National Park were quickly placed in insulated coolers that contained water which was 5°C warmer than the incubation temperature. During transit to the laboratory (ca. 2 h), the samples cooled slightly, but our procedure ensured that the samples remained within 5°C of the desired incubation temperature. In the laboratory, the samples were transferred to dark incubators that matched the desired incubation temperature. Methane was measured in the headspace at intervals as described below. Vials of samples from the Icelandic hot spring were sealed under N_2. For potential sulfate reduction rate assays, the vials were preincubated to permit equilibration to the desired temperature for incubation in the natural thermal effluent. Approximately 1 μCi of $Na_2{}^{35}SO_4$ (New England Nuclear Corp.; 783 mCi/mmol on 1 March 1979) from a sterile, anoxic stock solution was added. After 110 min of incubation, 0.2 ml of 2% (wt/vol) zinc acetate was added to fix $H_2{}^{35}S$, and 0.2 ml of Formalin was then immediately added to poison the vial. For methane production studies (Icelandic samples), approximately 2 μCi of $NaH^{14}CO_3$ (New England Nuclear; 44.5 mCi/mmol) was added as described above. The vials were poisoned at various intervals as described above. Temperatures decreased about 10°C at the in situ incubation sites, owing to weather changes over a 3-day incubation period.

Analytical methods. Gas samples (0.2 ml) were removed from the headspace of vials with a helium-flushed Glaspak syringe (Becton, Dickinson & Co.) attached to a Mininert valve (Supelco). CH_4 and $^{14}CH_4$ levels were determined by gas chromatography-gas proportion analysis as described by Ward and Olson (27). Gas concentrations and radioactivity were quantified with a Spectra-Physics model 4100 computing integrator and Minigrator, respectively. The total amounts of CH_4 and $^{14}CH_4$ were calculated by comparison of the subvolume with the gas volume in the headspace.

$H_2{}^{35}S$ and $^{35}SO_4{}^{2-}$ levels were determined by distillation and liquid scintillation counting as described by Jørgensen (17). Sulfate was determined turbidometrically (1). The potential rate of sulfate reduction was calculated as follows: [(micromoles of $SO_4{}^{2-}$ per milliliter of spring water) × milliliters of spring water × dpm of $H_2{}^{35}S$ per vial/(dpm of $^{35}SO_4{}^{2-}$ per vial) × hours of incubation]. Since mixing the cores with hot spring water could have elevated the sulfate concentration within the mat, the rate was considered a potential rate.

All results were analyzed by one-way analysis of variance (18).

Autofluorescence microscopy. Blue-green autofluorescence was taken as presumptive evidence of methanogenic bacteria in methane-positive cultures (19). Autofluorescence was observed with a Leitz Ortholux II microscope equipped with an HBO-100 W mercury lamp that provided vertical UV light illumination through a Leitz B-cube excitation-emission filter.

Isolation of thermophilic, methanogenic bacteria. Enrichment, isolation, and maintenance of thermophilic, methanogenic bacteria was done in a basal medium (BM) that contained the following (per liter of distilled water; all chemicals were reagent grade): KH_2PO_4, 0.15 g; Na_2HPO_4, 1.05 g; NH_4Cl, 0.53 g; $MgCl_2 \cdot 6H_2O$, 0.20 g; cysteine-hydrochloride, 0.5 g; resazurin, 0.0001 g; and trace mineral solution, 10 ml. The trace mineral solution contained the following (grams per liter of distilled water): nitrilotriacetic acid, 1.5; $FeCl_2 \cdot 4H_2O$, 0.3; $MnCl_2 \cdot 4H_2O$, 0.1; $CoCl_2 \cdot 6H_2O$, 0.17; $ZnCl_2$, 0.1; $CuCl_2$, 0.02; H_3BO_3, 0.1; and sodium molybdate, 0.01. The pH of the medium was adjusted to 9.2, so that the final pH was about 7.1 (±0.1) after all additions had been made. The medium was boiled under 100% helium (Linde Div., Union Carbide Corp.) but dispensed (5 ml per tube) under 20% CO_2–80% H_2 (Linde Div.) (15). All additions to the autoclaved medium were from sterile, anoxic stock solutions. $Na_2S \cdot 9H_2O$ was added to a final concentration of 0.03% (wt/vol). Since methanogenic bacteria have been shown to resist antibiotics that inhibit peptidoglycan synthesis (16), penicillin G or ampicillin was added to a final concentration of 300 μg/ml. BM was modified by adding 0.2% yeast extract and 0.2% Trypticase (BBL Microbiology Systems) (BM plus TYE) to check for heterotrophic contaminants. A contaminant organism was isolated on BM plus TYE by dilution to extinction. A turbid culture of the contaminant that was grown in BM plus TYE was sterilized by autoclaving, filtered through a 0.45-μm membrane filter (Millipore Corp.), and used to supplement BM (BM plus S) so that methanogenic bacteria could be isolated by dilution to extinction. It was later found that the supplementing culture could be replaced by vitamin B_{12}. Growth in the highest dilutions containing methane and blue-green autofluorescing cells was serially diluted until (i) all cells exhibited similar morphology and fluorescence and (ii) inoculation into BM plus TYE under a helium gas phase yielded no growth. Isolates were obtained at 50, 55, 60, and 65°C, with Octopus Spring mat samples obtained from sites at each of these temperatures as the inocula. To determine if growth could occur on formate, acetate, or methanol, we added each substrate separately to BM plus S (1% final concentration), with helium in the gas phase. In temperature studies, a 0.1-ml sample from a turbid culture which had been grown in BM plus S at the temperature at which it had been isolated was used to inoculate fresh BM plus S. Each isolate was incubated at 50, 55, 60, 65, 70, 75, and 80°C, and methane production was followed over time. In replicate tubes, the optical density at 660 nm of the isolates after 70 h of incubation relative to that of uninoculated medium was measured with a Varian model 635 or a Gilford model 250 spectrophotometer (1-cm light path). Tubes analyzed for optical density were flushed daily with 20% CO_2–80% H_2 and given about 2.5 atm (253 kPa) of the CO_2-H_2 gas mixture via a Glaspak syringe (10 ml) fitted with a Mininert valve. Sulfide was added after 48 h (0.05 ml of a sterile anoxic 3% $Na_2S \cdot 9H_2O$ solution) because it was presumably lost through daily gas headspace flushings.

RESULTS

Methanogenesis in Yellowstone National Park mats. As in earlier work (26), methane production measured within 48 h after the mats were sampled was considered representative of naturally occurring methanogenesis because extended incubation could result in exponential methanogenesis, presumably due to enrichment of methanogenic bacteria. Initial experiments were designed to determine the region of greatest methanogenesis along the thermal gradients of several hot spring microbial mats. Methane production in each of the springs surveyed was greatest at temperatures at least 13°C below the upper temperature limit of the mat (Fig. 1). The best temperatures for methane production were between 50 and 60°C. Methane production was significantly higher at the temperature of maximum methanogenesis than it was at other temperatures ($P \leq 0.048$), except when Weigert Channel mat methanogenesis at 55°C was compared with that at 60°C. Significant methanogenesis above 60°C occurred only in the Wiegert Channel mat.

When samples collected at the 51 or 62°C site in the Octopus Spring mat were incubated at other temperatures, methane production was greatest at 70°C ($P \leq 0.002$) (Fig. 2). Similar results were observed for samples of the Wiegert Channel mat (data not shown). Although some methane production was observed in samples incubated at 75°C, this was most likely methane production that occurred before the samples could be transferred to higher incubation temperatures. Time courses of methane production showed no further increases for samples incubated at 75°C for 3 days. Incubation at 80°C inhibited methane production in all samples. Incubation for several days, a period presumably sufficient to deplete immediate methane precursors, did not alter the results. Thus, anaerobic processes involved in the conversion of algal-bacterial material to methane at 50 to 60°C regions appeared to be most active at 70°C.

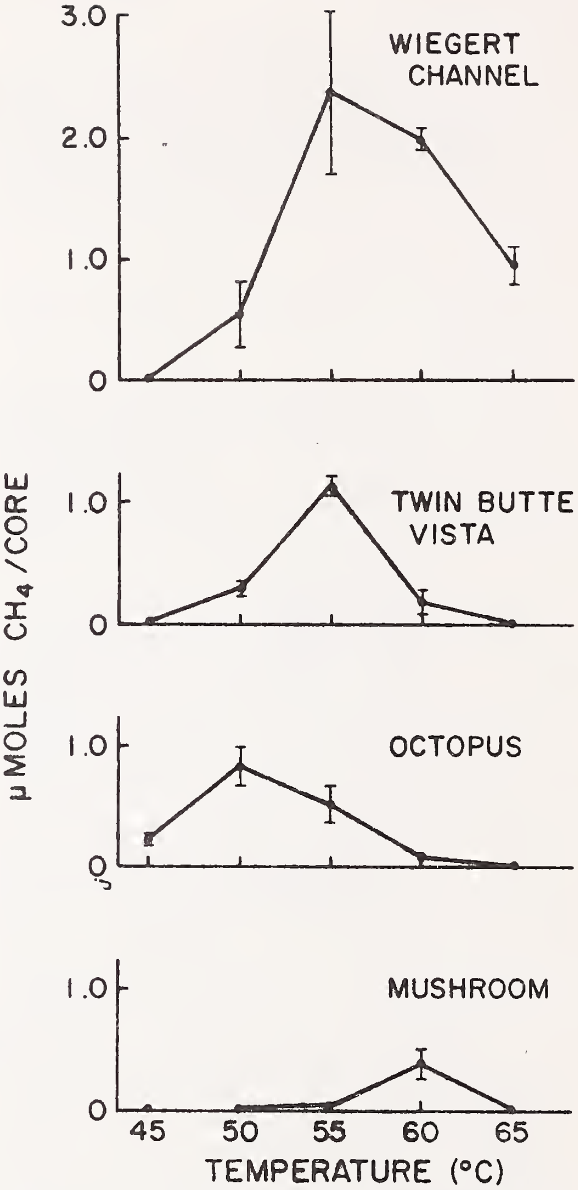

FIG. 1. Temperature distribution of methanogenesis in Yellowstone National Park hot spring microbial mats (methane produced after 17 to 20 h). Bars represent ±1 standard error ($n = 3$).

Pure cultures of methanogenic bacteria were obtained at incubation temperatures of 50, 55, 60, and 65°C from Octopus Spring mat samples obtained at these temperatures. All methanogenic isolates were long, irregular, gram-positive, rod-shaped bacteria which exhibited blue-green autofluorescence. Hydrogen, but not formate, acetate, or methanol, served as an energy source for growth. All methanogenic isolates showed the greatest growth and methane production at 65°C (Fig. 3) (P values were ≤ 0.05, except when methane production between 60 and 65°C were compared). Methane production and growth occurred when all isolates, except the strain obtained at 50°C, were

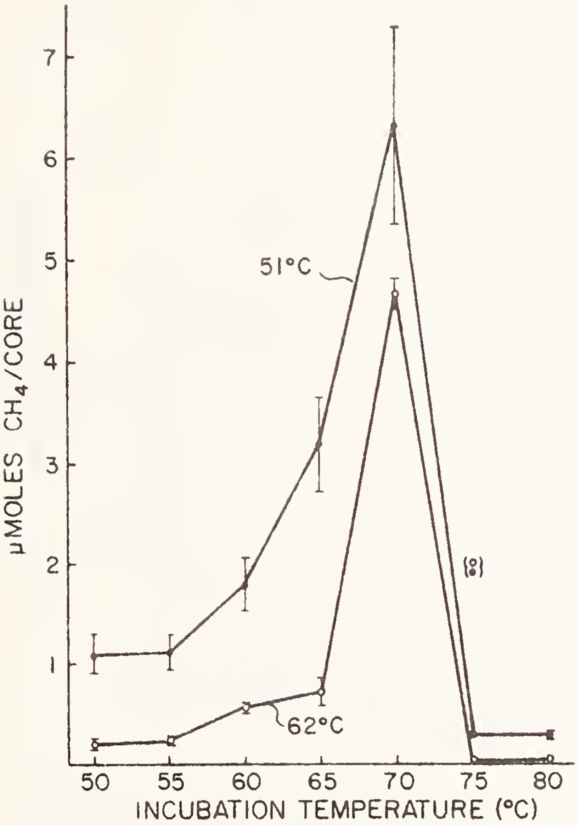

FIG. 2. Effect of temperature on methanogenesis in Octopus Spring algal-bacterial mat samples collected at 51 and 62°C sites (methane produced after 17 to 26 h). Bars represent ±1 standard error ($n = 6$). Points in parentheses are real amounts of methane detected at 75°C, but no increase in methane was detected after the first reading.

incubated at 70°C. No growth or methane production was observed at 75 or 80°C.

Sulfate reduction in the Icelandic mat. Methanogenesis and reduction of $NaH^{14}CO_3$ to $^{14}CH_4$ was not observed until days 2 and 3 of incubation of mat samples obtained at 50°C. However, $Na_2{}^{35}SO_4$ was rapidly reduced to $H_2{}^{35}S$. Potential rates of sulfate reduction were greatest at 50°C ($P \leq 0.002$) (Fig. 4), which was 10°C lower than the maximum temperature for mat formation. When samples collected from 50°C sites were incubated at various temperatures, the potential sulfate reduction rates at 60°C were found to be significantly higher than were those at all other temperatures except 55°C ($P \leq 0.028$), at which variance was uncommonly high. Production of $H_2{}^{35}S$ occurred at 75°C but not at 80°C.

DISCUSSION

Optimum temperatures for methane production in low-sulfate springs ranged from 50 to 60°C. Methanogenesis above 60°C was only significant in the artificial mat at Wiegert Channel. This is consistent with earlier reports on anaerobic decomposition (13) and methanogenesis (26) in Octopus Spring. Similar results were found for potential sulfate reduction rates in an Icelandic hot spring microbial mat. In this mat, the elevated level of sulfate presumably causes a dominance of sulfate reduction over methane production in the terminal step of anaerobic decomposition. This was suggested by a lag of 1 to 2 days before the onset of methanogenesis or conversion of $H^{14}CO_3{}^-$ to $^{14}CH_4$, as was seen in the high-sulfate Bath Lake microbial mat of Yellowstone National Park (27). These observations suggest that anaerobic decomposition in hot spring microbial mats is, in general, maximal at 10 to 23°C below the upper temperature for mat formation.

Methanogenesis in samples collected from areas of moderate temperature (50 to 62°C) in low-sulfate springs could be increased by incubation at higher temperatures and was optimal at 70°C, which is near the upper temperature for mat formation in Yellowstone National Park springs. This suggested that methanogenic bacteria are not optimally adapted to specific temperatures within the thermal gradients of the mats (73 to 40°C). To study the temperature relations of methanogenic bacteria directly, we obtained isolates at 50, 55, 60, and 65°C sites. Except for the vitamin B_{12} requirement, these isolates appeared to be similar to *M. thermoautotrophicum* (35), which was the dominant methanogenic bacterium isolated from a 65°C site at Octopus Spring (33). All isolates showed optimal growth and methanogenesis at 65°C, which was consistent with the results of temperature transfer experiments with natural mat material. Similar results reported for *M. thermoautotrophicum* isolated from a 65°C region of this mat led to the suggestion that methanogenic bacteria have adapted to the temperatures at which they are found (33). However, the inability of the isolate obtained at 50°C to grow or produce methane at 70°C was the only indication of temperature adaptation. A single-temperature strain of *M. thermoautotrophicum* appeared to exist in Octopus Spring mat samples obtained at temperature between 50 and 65°C.

The lack of specialization with respect to temperature may be generally true for anaerobic decomposition processes. Sulfate reduction was most rapid at 50°C in an Icelandic microbial mat, but incubation of 50°C samples at 60°C, the upper temperature for mat development, increased the rate. Although we have no direct evidence, it also seems likely that fermentative bacteria in the Yellowstone National Park mats have a similar preference for temperatures near the upper temperature for mat development (65

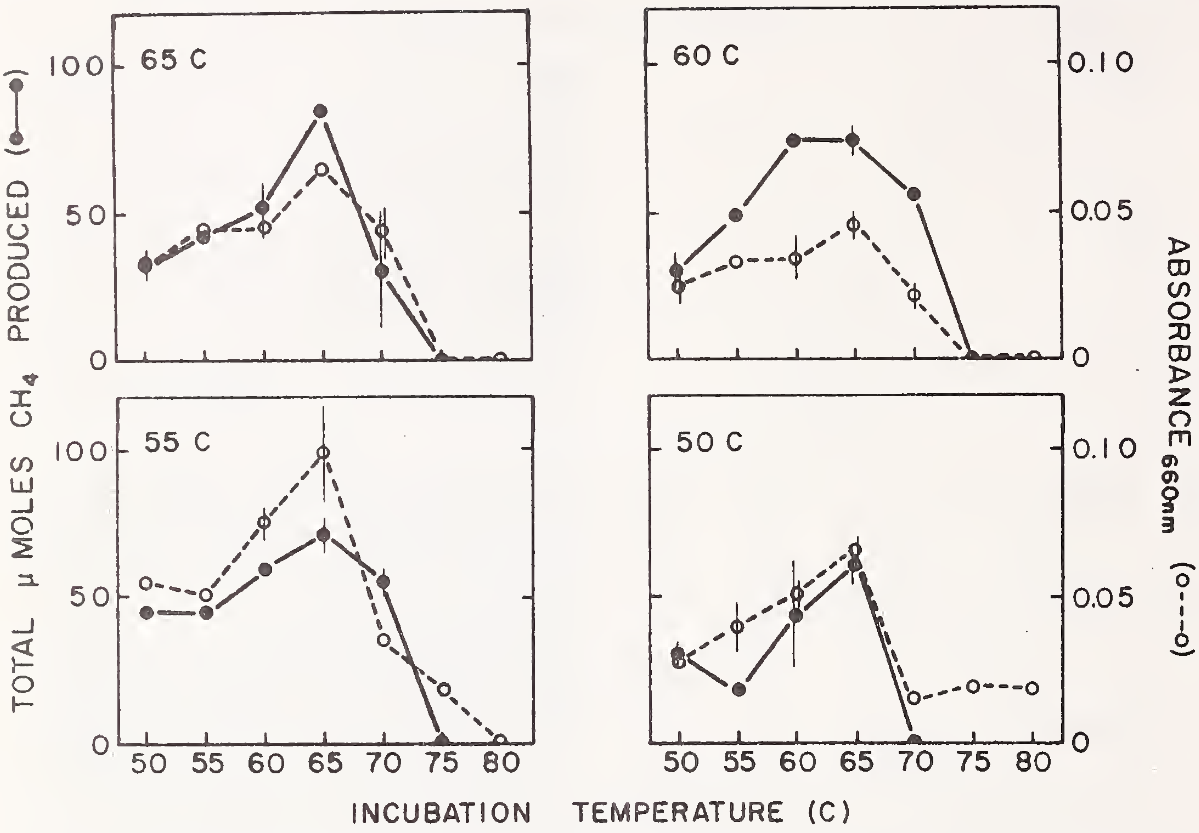

FIG. 3. Growth (expressed as absorbance at 660 nm) of and methanogenesis by methane-producing bacteria isolated from Octopus Spring (measured at 72 h). Isolates were obtained at 50, 55, 60, and 65°C regions and incubated at the temperatures indicated. Bars represent ±1 standard error when larger than the symbol ($n = 2$).

to 73°C). This is indicated by the dependence of methanogenesis on a continuous supply of hydrogen during dark incubation over several days (23). Anaerobic fermentative bacteria recently isolated from hot spring waters, sediments, and microbial mats near or in Octopus Spring show optimal growth at 65 to 70°C (3, 28, 33, 34). One of these isolates was obtained from a 45 to 50°C site (28).

It was interesting that sulfate reduction occurred in the Icelandic mat samples incubated at 75°C. This observation was not due to temperature fluctuations during incubation. Rozonova and Khudyakova (22) reported the isolation of a thermophilic, sulfate-reducing bacterium which has an upper temperature limit at 85°C but grows optimally at 65°C. The observation that sulfate reduction in samples obtained at 50°C was maximal at 60°C may indicate that sulfate-reducing bacteria of Icelandic springs have adapted to the maximum temperature of the mat ecosystem they inhabit. In this regard, it is also interesting that a strain of *M. thermoautotrophicum* isolated from a 57°C site in a nearby Icelandic mat grows better at 58°C (lower than the optimum temperature for other strains of *M. thermoautotrophicum*) than it does at 62 or 65°C (4). *Methanothermus fervidus* was recently isolated from an Icelandic hot spring but appears to be adapted to temperatures well above those at which microbial mats form in Iceland (24).

Since the bacteria involved in anaerobic decomposition in these systems prefer to grow near the upper temperature limit for mat development, some factor other than temperature must account for the fact that maximum methanogenesis and sulfate reduction occurred below the preferred temperature. Doemel and Brock (13) and Ward (26) suggested that decomposition is most active at temperatures at which the production of organic matter is most active (5, 7). Correlation between primary production of organic matter and methanogenesis in Octopus Spring was also observed in this study (K. A. Sandbeck and N. P. Revsbech, unpublished data). Primary production of organic matter in the Icelandic microbial mat was also found to be maximal at temperatures well below 60°C (unpublished data). These observations suggest that methanogenesis and sulfate reduction are limited by the supply of organic matter available for decomposition, the amount of which is related to the rate of mat formation. However, Ward (26) and Zeikus et al. (33) noted that hydrogen accu-

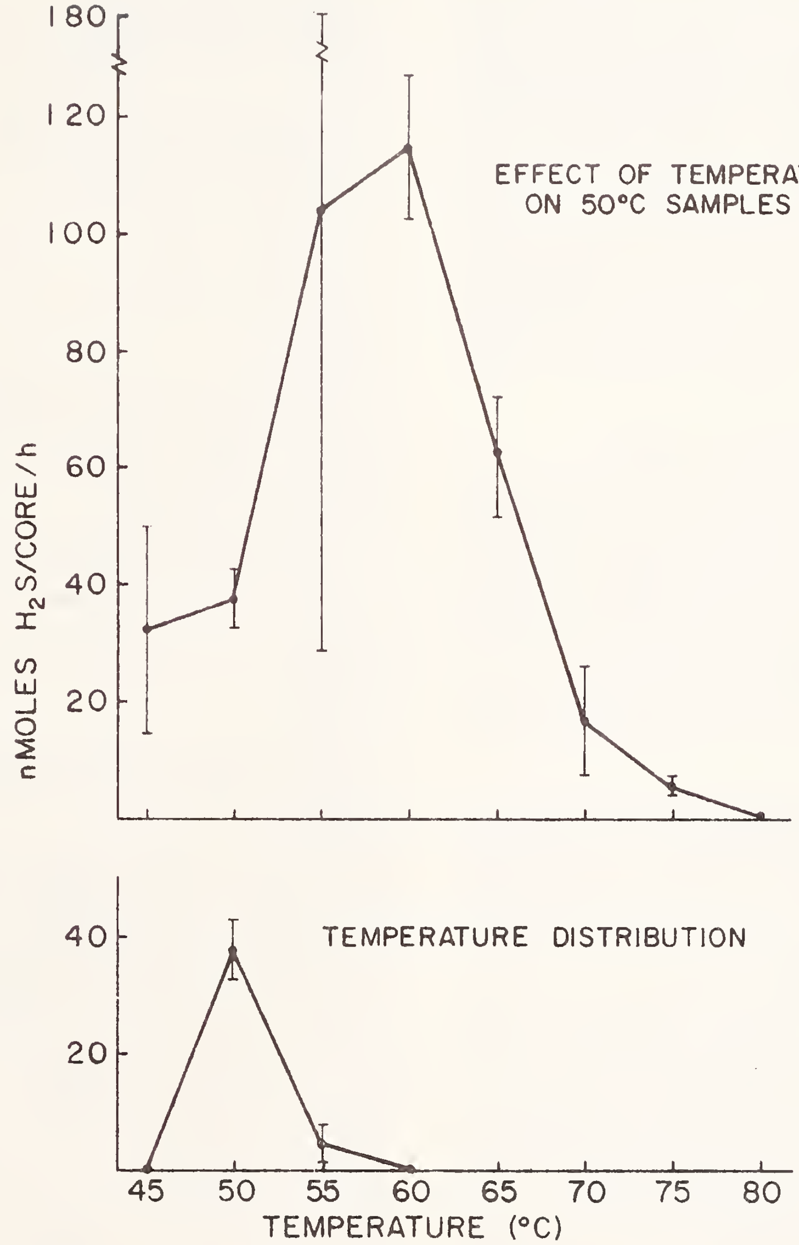

FIG. 4. Temperature distribution of potential sulfate reduction rate and effect of incubation temperature on potential sulfate reduction rate in samples collected at 50°C regions in an Icelandic hot spring microbial mat. Bars represent ±1 standard error (*n* = 2).

mulates in samples collected in Octopus Spring and incubated at 65°C or above. This suggests that fermentative H_2 production proceeds but is less tightly coupled to methanogenesis at the high-temperature extremes of the mat. Mat samples collected at temperatures near the upper extremes of the mat show methanogenesis with exponential kinetics after a lag when incubated anaerobically in darkness for several days (26). This suggests that the factors which are responsible for keeping the numbers of methanogenic bacteria and methanogenesis low and uncoupled from H_2 production are altered during dark, anoxic incubation. The subsequent enrichment of methanogenesis suggests that these factors (possibly O_2 or Eh) are more important than the supply of methanogenic substrates provided by decomposition of the sample.

Of obvious interest is the potential application of natural thermophilic microflora to high-temperature anaerobic conversions. Acclimation of mesophilic fermentations to temperatures above 60°C has been found to be difficult (9, 12, 25). Although maximum decomposition of low-sulfate mats to methane was restricted to temperatures of 50 to 60°C for ecological reasons, the microflora involved was most active at temperatures of 65 to 70°C. However, the application of natural thermophilic microflora to conventional thermophilic waste conversion processes may be difficult because acetate consumption is primarily mediated by phototrophic microorganisms rather than by acetate-using methanogenic bacteria in the algal-bacterial mats (23) and because of the well-known association between accumulation of volatile fatty acids and digestor failure (30). The only thermophilic, acetate-using, methanogenic bacterium which has been described was only able to grow at temperatures below 60°C (36) and was not observed in enrichments of the Octopus Spring mat (S. H. Zinder, personal communication).

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